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PI: Dr. Kristy M. Ainslie, The Ohio State University Co-I: Dr. Eric M. Bachelder, The Ohio State University Contractors: Dr. Andrea Keane-Myers, Naval Medical Research Laboratory (NMRC) Dr. Kevin Schully, NMRC

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14. ABSTRACT
For many diseases conferring rapid protection against an intracellular pathogen is required to prevent pathogen related death. To this end, we have developed a microparticulate vaccine carrier comprised of the novel polymer, acetalated dextran (Ac-DEX). Ac-DEX is an aptly designed polymer for vaccine applications because it's base material is FDA approved dextran and it has acid sensitivity for triggered release inside the phagosome, tunable degradation that can range from hours to months, and enhanced MHC I & II presentation with subunit antigen, compared to other biomaterials. In microparticulate form it can be used to passively target dendritic cells through

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DARPA 7-Day Challenge Final Report

ABSTRACT

For many diseases conferring rapid protection against an intracellular pathogen is required to prevent pathogen related death. To this end, we have developed a microparticulate vaccine carrier comprised of the novel polymer, acetalated dextran (Ac-DEX). Ac-DEX is an aptly designed polymer for vaccine applications because it's base material is FDA approved dextran and it has acid sensitivity for triggered release inside the phagosome, tunable degradation that can range from hours to months, and enhanced MHC I & II presentation with subunit antigen, compared to other biomaterials. In microparticulate form it can be used to passively target dendritic cells through size exclusion. We have formulated particles encapsulating recombinant protective antigen (rPA) or lysate (Francisella novicida, Burkholderia pseudomallei strain 1026) and the TLR 7/8 agonist resiquimod. Vaccination at 0 and 7 days (sub-Q) results in high levels of antigen specific antibodies for encapsulated formulations, compared to rPA + alum. Additionally, A/J mice (n=10) were aggressively challenged intratracheally with Bacillus anthracis (Sterne Strain) on day 14, 21 and 28, with survival in groups with encapsulated and/or free rPA and resiquimod. Studies were also performed with Ac-DEX microparticles encapsulating F. novicida or B. pseudomallei lysate. Balb/C (n=10 for F. novicida and n=25 for B. pseudomallei) mice were vaccinated on 0 and 7 days (sub-Q) with i.p. challenge on day 14. For the B. pseudomallei evaluation, we had delay onset to death in several groups, with post-mortem liver, spleen and blood in three of the nine experimental groups. Overall we have shown the efficacy of Ac-DEX microparticles for rapid vaccination against two intracellular bacterial pathogens.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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3. Schully KL, Sharma S, Peine KJ, Pesce J, Elberson MA, Fonseca ME, Prouty AM, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. Rapid protection against Francisella novicida via vaccination with acetalated dextran microparticles encapsulating cell lysate and resiquimod. In preparation.
4. Schully KL, Sharma S, Peine KJ, Fonseca ME, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. Microencapsulated vaccine for rapid and memory protection against highly virulent strain of Burkholderia pseudomallei. In preparation.
5. Brackman DJ, Gallovic M, Schully KL, Peine KJ, Borteh H, Bachelder EM, Keane-Myers A, Ainslie KM. Encapsulated resiquimod for Burkholderia thailandensis infection. In preparation.

10.2 Oral Presentations

1. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. OMICS Vaccines & Vaccination, Chicago, IL, July 2013.
2. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. AIChE, Pittsburgh, PA, Nov 2012.

10.3 Poster Presentations

1. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. AIChE, Washington, DC, Nov 2013.
2. Deanna J. Brackman, Hassan M. Borteh, Matthew D. Gallovic, Kevin J. Peine, Eric M. Bachelder, Kristy M. Ainslie. Encapsulated Resiquimod as a Treatment for Visceral Leishmaniasis. The Ohio State University College of Pharmacy Research Day, Columbus, OH. 2013
3. Kevin L Schully, Sadhana Sharma, Kevin J Peine, John Pesce, Margaret A Elberson, Mariko E Fonseca, Angela M Prouty, Matthew G Bell, Hassan Borteh, Matthew D Gallovic, Eric M Bachelder, Andrea Keane-Myers, Kristy M Ainslie. Rapid Vaccination Using an Acetalated Dextran Microparticulate Subunit Vaccine Confers Protection Aginst Triplicate Challenge by Bacillus Anthracis. College of Pharmacy Research Day, Ohio State University, Columbus, OH, April 2013.
4. Eric M. Bachelder, Sadhana Sharma, Kevin Schully, John T. Pesce, Andrea Keane-Myers, Kristy M. Ainslie. Novel Polymeric System for Rapid Vaccination. Gordon Conference Chemical & Biological Terrorism Defense, Mar 2011.
5. Eric M. Bachelder, Sadhana Sharma, Kevin Schully, John T. Pesce, Andrea Keane-Myers, Kristy M. Ainslie. Novel Polymeric System for Rapid Vaccination. Chemical and Biological Defense Science and Technology (DTRA), Nov 2011.

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03/17/2014	3.00	Schully KL, Sharma S, Peine KJ, Pesce J, Elberson MA, Fonseca ME, Prouty AM, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. . Rapid protection against Francisella novicida via vaccination with acetalated dextran microparticles encapsulating cell lysate and resiquimod. , (05 2014)
03/17/2014	4.00	Schully KL, Sharma S, Peine KJ, Fonseca ME, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. . Microencapsulated vaccine for rapid and memory protection against highly virulent strain of Burkholderia pseudomallei. , (12 2014)

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Books

Received Paper

TOTAL:

Patents Submitted

Patents Awarded

Awards

NA

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Kristy Ainslie	0.25	
Eric Bachelder	0.50	
FTE Equivalent:	**0.75**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Deanna Brackman	0.00	Other (Please Specify)
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 1.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 1.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 1.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 1.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 1.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:..... 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Sadhana Sharma	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

Please see attached report.

Technology Transfer

DARPA 7-Day Challenge Final Report

PI: Dr. Kristy M. Ainslie, The Ohio State University
Co-I: Dr. Eric M. Bachelder, The Ohio State University

Contractors:
Dr. Andrea Keane-Myers, Naval Medical Research Laboratory (NMRC)
Dr. Kevin Schully, NMRC

Date: May 2013

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1. Abstract

For many diseases conferring rapid protection against an intracellular pathogen is required to prevent pathogen related death. To this end, we have developed a microparticulate vaccine carrier comprised of the novel polymer, acetalated dextran (Ac-DEX). Ac-DEX is an aptly designed polymer for vaccine applications because it's base material is FDA approved dextran and it has acid sensitivity for triggered release inside the phagosome, tunable degradation that can range from hours to months, and enhanced MHC I & II presentation with subunit antigen, compared to other biomaterials. In microparticulate form it can be used to passively target dendritic cells through size exclusion. We have formulated particles encapsulating recombinant protective antigen (rPA) or lysate (*Francisella novicida, Burkholderia pseudomallei* strain 1026) and the TLR 7/8 agonist resiquimod. Vaccination at 0 and 7 days (sub-Q) results in high levels of antigen specific antibodies for encapsulated formulations, compared to rPA + alum. Additionally, A/J mice (n=10) were aggressively challenged intratracheally with *Bacillus anthracis* (Sterne Strain) on day 14, 21 and 28, with survival in groups with encapsulated and/or free rPA and resiquimod. Studies were also performed with Ac-DEX microparticles encapsulating *F. novicida* or *B. pseudomallei* lysate. Balb/C (n=10 for *F. novicida* and n=25 for *B. pseudomallei*) mice were vaccinated on 0 and 7 days (sub-Q) with i.p. challenge on day 14. For the *B. pseudomallei* evaluation, we had delay onset to death in several groups, with post-mortem liver, spleen and blood in three of the nine experimental groups. Overall we have shown the efficacy of Ac-DEX microparticles for rapid vaccination against two intracellular bacterial pathogens.

2. Introduction

Vaccines are arguably the most effective agents developed for the prevention of disease, disability, death and the control of health care costs. However, for many lethal infections, the time course from pathogen exposure to death is shorter than the time required for induction of effective humoral or cellular-mediated immunity. For example, pulmonary *Bacillus anthracis* (*B. anthracis*) can cause death within one week of infection while the current vaccine requires multiple doses (3-6) and multiple months (6-18 months) to achieve protective titers[1]. A typical method of vaccination is based on the use of live attenuated viruses or bacteria, but such pathogens present risks to humans, precluding their widespread use. Thus, new vaccination strategies are urgently needed[2]. In contrast to live attenuated vaccines, sub-unit vaccines which incorporate protein antigens have considerable promise because of their low toxicity and broad applicability. Sub-unit based vaccines are currently being investigated in clinical trials for the treatment of AIDS, hepatitis C and cancer[3]. However, antigen delivery problems have limited their clinical effectiveness, and new delivery vehicles are greatly needed that can enhance the potency of sub-unit based vaccines. Sub-unit vaccines must mimic several biological consequences of natural infections to generate an effective adaptive immune response. In particular, two key events must occur for successful vaccination: (1) delivery of the antigen to antigen presenting cells (APCs) and (2) activation of the APCs in a manner that mimics the presence of an infectious pathogen.

Alternatives exist for the enhancement of protein based vaccines. One method is to increase the efficiency of the immunological synapse[4], specifically increasing the efficacy of antigen presentation to T cells. For example, dendritic cells (DCs) or macrophages can be pulsed with high loads of pathogen antigens[5], transfected with plasmids[6], transfected with mRNA[7] or infected with viral vectors[8]. At present, the majority of these approaches rely on *ex vivo* manipulation of isolated DCs. These methods are expensive, require extensive preparation including *ex vivo* pre-expansion of patient's dendritic cells, and require extensive knowledge of the vector's pathogenesis and epitopes that when immunized against will result in an effective immune response when challenged by the specific vector. Even though these methods are effective, it is desired to come up with an alternative method to target DCs *in situ* with proteins associated with bioterrorism agents.

We have explored another approach for the loading of DCs with antigen while eliminating the need for *ex vivo* manipulation of DCs. We began designing and synthesizing particulate carriers capable

of both carrying a payload of antigen and also of targeting and activating DCs *in vivo*. Most cell types *in vivo* lack phagocytosis capabilities and are incapable of internalizing particulate carriers. Due to the phagocytic properties of DCs, particulate carriers are ideal for "passively targeting" DCs *in vivo.* Antigens can be encapsulated in a particulate carrier, injected *in vivo*, and reside at the site of injection site until being phagocytosed by resident DCs. This mechanism allows DCs to be loaded with antigen *in vivo*, instead of the *ex vivo* techniques that are currently used. An ideal vehicle for antigen-based vaccines must be capable of delivering antigen into antigen presenting DCs while simultaneously activating toll-like receptors (TLRs). This requires the development of carriers that can target DCs and ensure the delivery of antigens for efficient class I and class II antigen presentation. In addition, vaccine delivery vehicles should also possess specific ligands (such as TLR agonists) for efficient uptake by, and stimulation of APCs. CpG, a TLR9 agonist [9-11], and poly (I:C), a TLR 3 agonist [12], have been co-encapsulated in polymeric carriers with proteins resulting in an enhancement in both $CD4^+$ and $CD8^+$ T cell activation against the encapsulated protein.

The most common polymer used as a carrier for drug delivery applications is poly(lactic-*co*-glycolic acid) (PLGA) due to its inherent biodegradability and low toxicity. However, for vaccine applications, the utility of PLGA may be limited. For example, it may be desirable to have a material that is sensitive to the acidic environment present in the phago-lysosomal compartments of macrophages and dendritic cells, which have an approximate pH of 5 [13]. An acid-sensitive material allows for the expedient delivery of protein to the cross-presentation machinery present in the phagosome [14,15]. In addition, as PLGA degrades, an acidic microenvironment is created by the accumulation of lactic and glycolic acid. Prolonged exposure to acidic environments may be harmful to the stability of recombinant proteins used in vaccines, e.g., tetanus toxoid and diphtheria toxoid [16]. Due to these inherent problems with PLGA, new polymeric carriers are desired for vaccine applications.

To address this issue of pH sensitivity in polymeric carriers, we developed the polymer Acetalated Dextran (Ac-DEX), a new polymer that is made through the acetalation of dextran, a homopolysacharide of glucose, which renders the modified polymer soluble in common organic solvents, but completely insoluble in water [17]. These properties allow for the facile processing of Ac-DEX into microparticles encapsulating antigens for vaccine applications through the use of standard emulsion techniques. Due to the acid-catalyzed hydrolysis of acetals, microparticles made from Ac-DEX are pH-sensitive and degrade quicker in lysosomal pH (~ 5) compared to physiological pH (7.4). Beyond its performance in vaccine formulations, this material is extremely promising due to its simplicity, scalability, tunability, and other intrinsic properties (see Figure 1 and preliminary data below). This pH-sensitivity allows for a significant increase in both MHC I and MHC II presentation compared to other biomaterials [18]. Degradation of Ac-DEX does not lead to acidic by-products, and therefore Ac-DEX should be more suitable for pH-sensitive antigens compared to other materials. In addition, we have demonstrated that pH sensitive particles enhance the efficacy of antigen-based vaccines by increasing CD4+ and $CD8^+$ T-cell activation[18-20]. We have also shown that these particles are superior to PLGA-based particles and that the rate of particle degradation can significantly affect their biological properties.

This research is a vast improvement of existing technology. The **first improvement**, is the use of Ac-DEX particles, a synthetically made biofriendly polymer based on dextran[18,19] which has shown a drastic improvement over existing technology in enhancing immune responses. The **second improvement**, is the encapsulation of resiquimod, a highly stimulating adjuvant is typically used as a topical cream which limits its application[21]. The **third improvement**, the experiments described will use lysate from an unknown pathogen, which requires little prior knowledge of the pathogen. As long as a pathogen can be grown *in vitro*, no further characterization is required in developing a vaccine. The **fourth improvement**, is the particulate carrier targets DCs *in vivo* which will not require the *ex vivo* expansion of DCs. The **fifth improvement**, is the immune response against an antigen is drastically enhanced through specific DC targeting and resiquimod encapsulation, decreasing the time required to generate an effective immune response. In addition, targeting DCs *in vivo* could lead to dose sparing which would limit the potential of adverse effects present in other vaccines[22].

Data presented in this introduction was not supported by this grant and is solely presented

to provide background.

1.1 Humoral Response

Current vaccines against many bioterrorism agents take too long in inducing an effective humoral and cellular-mediated immunity. For example, pulmonary *Bacillus anthracis* (*B. anthracis*) can cause death within one week of infection while the current vaccine requires multiple doses (3-6) and multiple months (6-18 months) to achieve protective titers[1]. Thus, new vaccination strategies are urgently needed[2].

The following research will drastically increase the efficacy of protein based vaccines which will provide protection against a pathogen in a much shorter time frame. In order to do this, we have designed and synthesized particulate carriers capable of both carrying a payload of antigen and also of targeting and activating DCs *in vivo*. Due to the phagocytic properties of DCs, particulate carriers are ideal for "passively targeting" DCs *in vivo*. Antigens can be encapsulated in a particulate carrier, injected *in vivo*, and reside at the site of injection site until they are phagocytosed by resident DCs. We have demonstrated that pH sensitive particles, with or without adjuvants, enhance the efficacy of antigen-based vaccines by drastically increasing CD4+ and $CD8^+$ T-cell activation. In addition, targeting DCs *in vivo* could lead to dose sparing which would limit the potential of adverse effects present in other vaccines[22].

1.2 Rapid Antigen Development

Current vaccines require extensive knowledge of the pathogen. As stated previously, a typical method of vaccination is based on the use of live attenuated viruses or bacteria. Extensive time and money is involved in developing an attenuated pathogen for a given disease. In addition, when using sub-unit vaccines (vaccines based on recombinant proteins associated with a pathogen), immunological epitopes that provide protection must be defined which requires extensive immunological assays. Once epitopes are defined, recombinant production of the defined protein must be performed which require time and money. In certain instances, an unknown virus or bioterrorism agent exposed to the general public or military personal could have cataclysmic results. These instances would necessitate the development of a vaccine in the quickest time possible.

The following approach will drastically decrease the time required in developing a vaccine against an unknown pathogen. In order to do this, the experiments described herein will use lysate from an unknown pathogen, which requires little prior knowledge of the pathogen. As long as a pathogen can be grown *in vitro*, no further characterization is required in developing a vaccine.

1.3 In vivo Targeting of Dendritic Cells

Current state of the art vaccine techniques require ex vivo manipulation of DCs. One method of increasing the efficacy of a vaccine is to increase the efficiency of the immunological synapse[4], specifically increasing the efficacy of antigen presentation to T cells. For example, DCs can be pulsed with high loads of pathogen antigens[5], transfected with plasmids[6], transfected with mRNA[7] or infected with viral vectors[8]. At present, the majority of these approaches rely on *ex vivo* manipulation of isolated DCs. These methods are expensive, require extensive preparation including *ex vivo* pre-expansion of patient's dendritic cells, and require extensive knowledge of the vector's pathogenesis and epitopes that when immunized against will result in an effective immune response when challenged by the specific vector. Therefore, it is desired to develop an alternative method to target DCs *in situ* with proteins associated with bioterrorism agents.

Our method of DC targeting does not require ex vivo manipulation of DCs. We have designed and synthesized a particulate carrier capable of both carrying a payload of antigen and also of targeting and activating DCs *in vivo*. Due to the phagocytic properties of DCs, particulate carriers are ideal for "passively targeting" DCs *in vivo*. Antigens can be encapsulated in a particulate carrier, injected *in vivo (*e.g. s.c., i.t., i.p.*)*, and reside at the site of injection site until being phagocytosed by resident DCs. This

mechanism allows DCs to be loaded with antigen *in vivo*, instead of the *ex vivo* techniques that are currently used.

Unlike most cell types *in vivo*, macrophages and dendritic cells (DCs) have phagocytic properties [23] allowing them to internalize particles larger than 100 nm. Particles of this size cannot be formulated with techniques like traditional spray drying [24]. Also, Ac-DEX formulations would not have the stability and storage issues associated with other technology like liposomes or micelles [25]. We have previously used passive targeting of Ac-DEX nanoparticles to deliver TLR agonists to DCs to increase activation. By incorporating an adjuvant, such as resiquimod (a TLR7 & 8 agonist) into microparticles, we can drastically increase pro-inflammatory cytokine production (IL-1β, IL-12p70, IL-6, and MIP 1α) of bone marrow derived DCs (BMDCs) compared to free imiquimod (i.e. dose sparing: less drug is needed for equal activation) [26] (Figure 1).

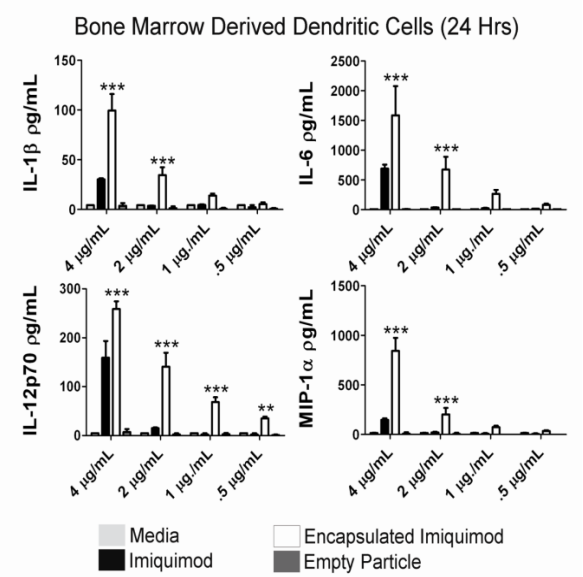


Figure 1: Dose Sparring of Imiquimod Ac-DEX particles
Encapsulation of imiquimod results in higher bone marrow derived dendritic cell activation.[27]

In addition to enhancement of adjuvant delivery, our results show a ten-fold increase in protein antigen uptake by phagocytic cells occurs when the protein is presented to the cell in a pH sensitive nanoparticle [28] (Figure 2).

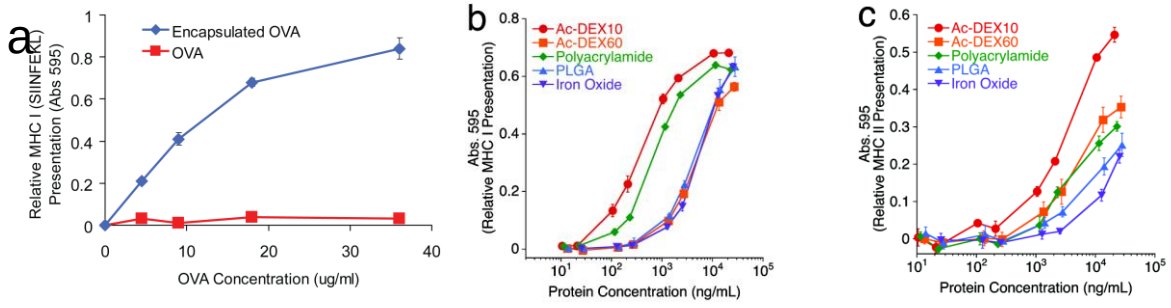


Figure 2: MHC presentation of protein antigen with Ac-DEX particles
(a) Relative MHC I presentation of DCs for OVA-containing Ac-DEX particles (encapsulated) and OVA in solution (OVA) (b) Relative MHC I presentation of DCs for OVA-containing Ac-DEX10, AC-DEX60, acid-degradable polyacrylamide, PLGA, and iron oxide particles. Quickly degrading materials (Ac-DEX10 and polyacrylamide) show presentation at significantly lower protein concentrations. (c) Relative MHC II presentation of DCs for OVA-containing particles.[18,29]

Overall, encapsulation has shown the potential to significantly increase drug activity, and allow dose sparing which would decrease the side-effects inherent with adjuvants. Also, through passive targeting, other more costly and time consuming methods, like *ex vivo* expansion of DCs are unnecessary since through facile application via subQ, i.t., or i.n. routes APCs can be specifically targeted. For these reasons, we will use Ac-DEX particles as a method to develop vaccines and treatments for bioterrorism agents.

1.4 pH Sensitive Biomaterial for Phagosomal Delivery

Current state of the art biomaterials are not pH sensitive. The most common polymer used as a carrier for drug delivery applications is poly(lactic-*co*-glycolic acid) (PLGA) due to its inherent biodegradability and low toxicity. However, for vaccine applications, the utility of PLGA may be limited. For example, it is desirable to have a material that is sensitive to the acidic environment present in the phago-lysosomal compartments of dendritic cells, which have an approximate pH of 5 [13]. An acid-sensitive material allows for the expedient delivery of protein to the cross-presentation machinery present in the phagosome [14,15]. In addition, as PLGA degrades, an acidic microenvironment is created by the accumulation of lactic and glycolic acid. Prolonged exposure to acidic environments may be harmful to the stability of recombinant proteins used in vaccines. Due to these inherent problems with PLGA, new polymeric carriers are desired for vaccine applications.

To address this issue of pH sensitivity in polymeric carriers, we developed the polymer Acetalated Dextran (Ac-DEX), a new polymer that is based on dextran[17]. This pH-sensitivity allows for a significant increase in both MHC I and MHC II presentation compared to other biomaterials [18]. Degradation of Ac-DEX does not lead to acidic by-products, and therefore Ac-DEX should be more suitable for pH-sensitive antigens compared to other materials. In addition, we have demonstrated that pH sensitive particles enhance the efficacy of antigen-based vaccines by increasing CD4+ and $CD8^+$ T-cell activation[18-20] and are superior to PLGA-based particles.

In addition to the potential of PLGA harming the immunogenic payload, it is also not ideally suitable for immunotherapy applications. It has been hypothesized that rapid vehicle degradation may be important for achieving efficient antigen cross-presentation from APCs [30]. Based on this, B3Z cells [31] were used to quantify MHC I presentation from BMDCs after incubation with OVA loaded particles made from samples of Ac-DEX (Figure 2) [13]. DCs cultured *in vitro* with particles encapsulating ovalbumin (OVA) have a substantial higher level of presentation of MHC I peptides compared to free OVA (Figure 2a). In Figure 2b, fast degrading Ac-DEX particles (denoted by Ac-DEX10) have a log difference in MHC I presentation compared to particle carrier systems that do not have pH sensitivity. The polyacrylamide nanoparticle in Figure 2b, is comprised of the monomer ***acrylamide***, and a pH sensitive crosslinker. Since this particle system contains acrylamide, which is a neurotoxin, it is not suitable for *in vivo* applications and serves only as a historical control [32]. In addition, the fast degrading Ac-DEX polymer can enhance MHC II presentation as well (Figure 2c). Finally, we have shown that *in vivo* our Ac-DEX nanoparticles can enhance CTL cell proliferation when compared to free protein (Figure 3). These data are consistent with what we have shown previously *in vivo* that pH sensitive nanoparticles enhance CTL activity against tumor cell lines expressing a specific antigen [32]. **Overall, encapsulating sub-unit antigens in our particles will increase the T-helper and Cytotoxic T-cell activity against the select agent antigens.** This technology is a vast improvement on current subunit vaccination strategies.

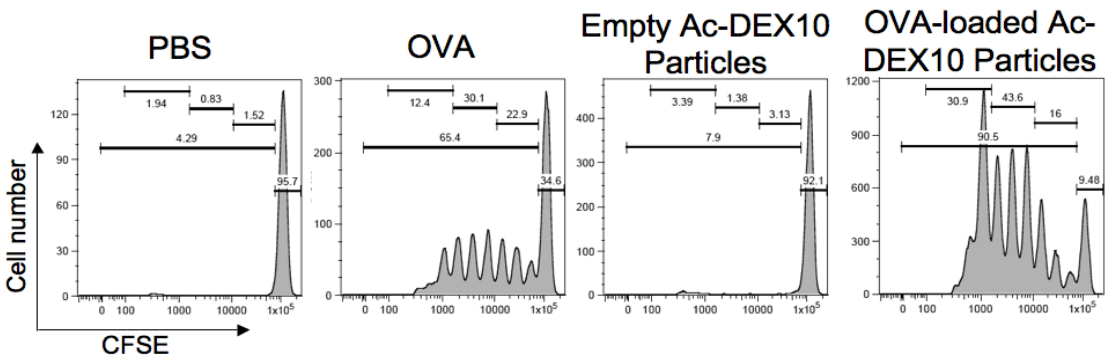


Figure 3: T cell proliferation with OVA Ac-DEX particles
Encapsulation of OVA in Ac-DEX10 particles significantly enhances T cell proliferation compared to free OVA *in vivo*.

1.5 Delivery of Adjuvants

Protein vaccinations have limited efficacy in vivo. Adjuvants can be mixed with the protein drastically increasing the immune response against the protein. However, immunological adjuvants approved for human use are very limited. Resiquimod is an adjuvant that is FDA approved for human use, has a very high activity, but is typically delivered as a topical cream.

We have incorporated resiquimod in our polymeric carrier system. We have preliminary data showing that the delivery of resiquimod in our polymeric carrier system drastically increases the efficacy of resiquimod.

Polymer	By-Product	Degradation Half-life
Ac-DEX[18,33]	Dextran, alcohol, & acetone	Tunable: hrs - months+
PLGA (also PLA and PGA)[34]	Lactic and/or glycolic acids	~2 months
poly-ε-caprolactone (PCL)[35]	Caproic and other acids	>3 months
Polyanhydrides[36]	Acid by-products	> 5 months

Table 1: Fully Degradable Biopolymers

1.6 Benign Polymer By-products

Application of Ac-DEX as a polymer carrier would serve as a shift in the current research of drug delivery vehicles. The application of poly-lactic-co-glycolic acid (PLGA), a polyester polymer, or its derivatives (poly-lactic or poly-glycolic), is ubiquitous with polymeric delivery. Polyesters all have acidic by-products which can shift the local pH (Table 1) causing cellular damage. For example, a change in the local pH of the pulmonary mucosa can lead to conditions like hyperosmolarity that can enhance bacterial invasion through lung epithelium [37]. In addition, the encapsulation of protein in PLGA results in a highly acidic environment (pH <3) around the encapsulated protein [38]. This low acidic environment during degradation can drastically denature the encapsulated protein. With the use of Ac-DEX, we offer a significant advantage in that the by-products are dextran and extremely low levels of acetone and an alcohol. Dextran has been used for years safely as a blood additive, and the alcohol released is much less than that observed with typical ethanol consumption (<<nM range) [18,33]. Acetone is at levels comparable to those observed with cellular metabolism. Indeed, *in vitro* assays indicate Ac-DEX is as safe as FDA approved PLGA (Figure 4). In addition, our polymer will not lower the pH of the surrounding aqueous environment, which would have the potential of harming pH sensitive antigens. By using Ac-DEX, a vaccine and therapeutic carrier can be developed without acidic, potentially harmful, degradation products.

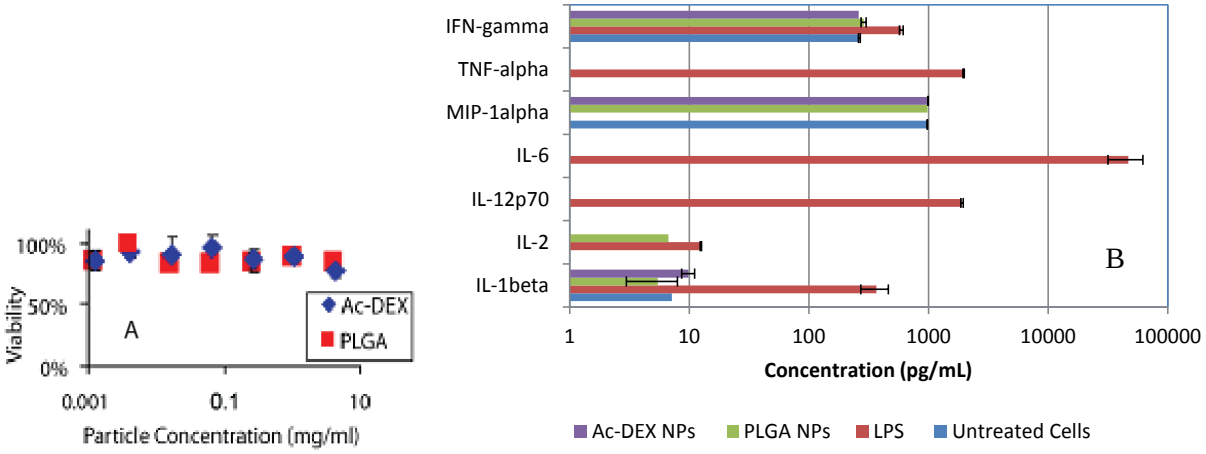


Figure 4: In vitro toxicity of Ac-DEX Particles
(A) Ac-DEX particles are compared to PLGA NPs. (B) NPs at 12.5 μg/mL and LPS at 1 μg /mL.

1.7 Tunability for Controlled Release

Modification of dextran with acetals results in the formation of both cyclic and acyclic acetals. These two different types of acetals have significantly different rates of hydrolysis in acidic conditions. We have shown that by varying the coverage of these two types of acetals, we can generate a library of materials with different degradation rates at acidic pH (Fig 4). From this library, we can generate particles that degrade at one or several different rates, resulting in precise and sustained release of adjuvant and antigen. Sustained release can result in a continuous stimulation of DCs, similar to a depot effect observed with use of CFA. Previous research has shown that the rate of antigen release from a polymeric emulsion can drastically affect the immune response against the encapsulated antigen [39-41]. **By exploiting the tunability of Ac-DEX degradation, sustained APC modulation, increased T cell presentation, and therapeutic controlled release can be achieved.** Once again this method is superior to the commonly used PLGA polymer. The degradation of PLGA cannot be modified and has one degradation rate that is on the order of weeks. Depending on the application we can design Ac-DEX to release the encapsulated cargo very quickly or very slowly making the Ac-DEX carrier system superior to existing technology.

Rxn Time	Cyclic Coverage	Degradation Time (hours)		
		pH 5.0	pH 6.9	pH 7.5
5 min	40	0.5	36	64
20 min	60	1.1	167	222
6 hr	80	17.2	1467	1134

Table 2: Degradation rates of Ac-DEX at different pHs
Degradation rates of Ac-DEX particles at three different pH conditions. Increasing reaction time leads to an increased number of cyclic acetals and therefore a longer degrading polymer.[18]

1.8 Stability of Encapsulated Protein

An ideal vaccine would be capable of being highly effective, and not require the cold chain process [42]. The World Health Organization recommends that all vaccines (excluding the polio vaccine) be stored at 4°C during distribution. However, from a military standpoint, the cold chain distributions for vaccines, especially in remote and isolated areas of deployment are hard to accomplish. Stability of proteins in polymeric particles has previously been characterized [43,44]. *Brucella abortus* extract was encapsulated in PLGA particles for up to one year at 40°C and 75% relative humidity. At this elevated

temperature, the *B. abortus* extract is still antigenic after six months. In addition, Sloat et al. has shown that PLGA particles encapsulating bovine serum albumin (BSA) are still capable of inducing an antibody response after storage at 37°C for 2.5 months. It has been noted that to guarantee the long-term stability of lyophilized protein, the glass transition temperature (Tg) of the protein formulation must be much higher than the planned storage temperature. This ensures that any molecular motion and associated degradation was minimized [45]. Ac-DEX is a highly crystalline bio-polymer that we have shown to have a Tg of approximately 160°C, while the Tg of PLGA is around 40-50°C. As an example of how stable are particles are compared to PLGA, we stored our particles in dry conditions at 45 °C for four days. At four days, PLGA particles begin to fuse together while Ac-DEX particles resist agglomeration. Furthermore, we have shown that enzyme activity is independent of storage temperature when the protein is encapsulated in Ac-DEX (Figure 5). Based on this data, we will potentially be able to develop a vaccine technology that can avoid the cold chain and resist elevated temperatures for storage.

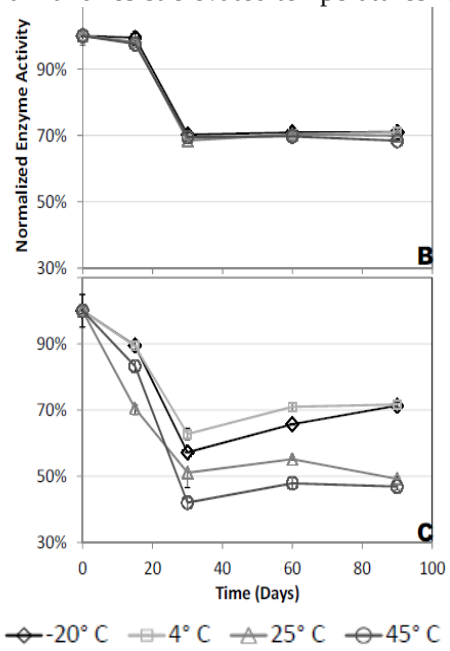


Figure 5: Enhanced storage of protein with Ac-DEX encapsulation
B) Enzyme activity of HRP encapsulated in Ac-DEX at 4 temperatures. (C) Enzyme activity of unencapsulated HRP.

1.9 Scalable Production of Particles

When synthesizing particles, there is a possibility of inconsistency of protein loading or adjuvant loading between different lots of particles. However, we have shown that using Ac-DEX we have had consistent protein loading of a model antigen. By using double emulsion techniques we were able to consistently obtain the same protein loading in different particle synthesis batches. Traditionally in a laboratory set-up, emulsion chemistry is used to make particles. This type of synthesis is suitable for immunizing mice, but is not scalable for treatment of larger mammals like humans. Finally, emulsion chemistry requires high energy input to form the emulsion, which could be damaging to proteins resulting in denaturation and loss of antigenicity [46,47].

In the pharmaceutical industry, particles are synthesized by using a spray dryer [48]. Spray drying uses a spray nozzle to disperse a slurry into a controlled drop size spray. While aerosolized, the spray is exposed to a hot drying gas that causes the solvent to evaporate quickly resulting in particle formation. Traditional spray drying, although scalable, produces particles larger than what is typically used for delivery to phagocytic cells. Electrospray particle synthesis is a type of spray drying, which is gentle and works by applying a uniform electrohydrodynamic force to break up liquids into fine jets, which provides

high encapsulation efficiencies at relatively low loadings, and does not cause protein denaturation, which can potentially enhance B cell responses. This method allows for an increased scalability and reproducibility that is not achieved with emulsion chemistry. As a proof of concept, we have electrosprayed Ac-DEX to form microparticles suitable to delivery to phagocytic cells (Figure 6). This method was not used for particle development in this proposal, but could be used for future work.

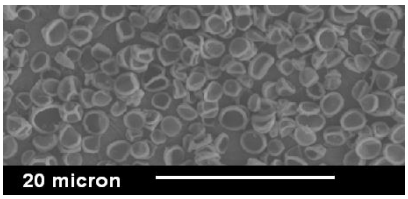


Figure 6: Electrosprayed Ac-DEX particles
SEM of electrosprayed Ac-DEX particles

1.10 Ac-DEX Particle Vaccines vs. Alum Vaccines

The current state of the art FDA approved adjuvant is Aluminum Hydroxide (Alum) [49]. The exact mechanism of how alum works *in vivo* has not been fully elucidated but recent research has shown that alum can alter DC biology [50]. Proteins (antigens) can bind to alum through electrostatic interactions [51]. This interaction depends on the pI and the type of alum that is being used. Therefore some proteins bind to alum better than others. Unfortunately, for vaccine applications, alum cannot co-encapsulate TLR agonists which would potentially increase the activation of DCs. In addition, it is known that alum vaccines cannot be lyophilized due to severe aggregation [52]. Therefore, most alum based vaccines must be stored between 2-8°C and have a limited shelf life. Finally, alum is not FDA approved for pulmonary delivery [53]. Ac-DEX particle technology can solve all the limitations of alum. particles can encapsulate a myriad of different proteins, can co-encapsulate TLR agonists, can be lyophilized for long term storage, and can be delivered to the lung [54].

2. Fabrication of Ac-DEX

2.1 Introduction

Ac-DEX is synthesized in a single step from a natural polymer, possesses a favorable toxicity profile, and can be processed into materials encapsulating either hydrophobic or hydrophilic payloads. Because of these favorable attributes, Ac-DEX-based materials may have significant advantages over other pH-sensitive or biocompatible materials currently used in biomedical applications.

2.2 Ac-DEX Synthesis

Ac-DEX was prepared with some modifications to the procedure described by Broaders et al.[18]. Lyophilized dextran (MW= 10,000 or 71,400) and pyridinium p-toluenesulfonate (0.0617 mmol) were dissolved in anhydrous DMSO and reacted with 2-methoxypropene (37 mmol) under nitrogen gas. After required reaction time, the reaction was quenched with triethylamine, precipitated in basic water (pH 9), vacuum filtered, and lyophilized for two days to yield a fluffy white solid. The product was then purified by dissolving it in ethanol (200 proof) and centrifuged (5 min, 10,000 x g, Beckman RA-21, Los Angeles, CA, USA). The supernatant was precipitated in basic water and lyophilized for two days to yield Ac-DEX. The fabrication of Ac-DEX is presented in Figure 8.

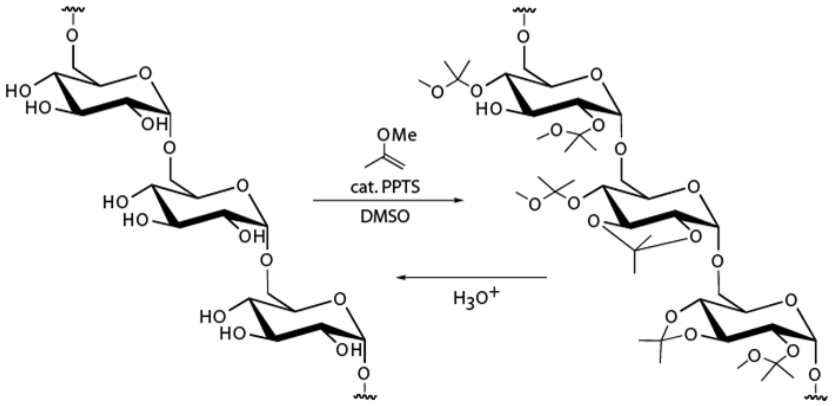


Figure 7: Ac-DEX Reaction Scheme
The reaction scheme to synthesize acetalated dextran (Ac-DEX) polymer.

2.3 Ac-DEX NMR Analysis

NMR analysis provided the relative cyclic:acyclic ratio of acetal groups of the Ac-DEX polymer. Ac-DEX was suspended in deuterium oxide and hydrolyzed with deuterium chloride. After two hours, the samples were read with a 300 MHz 1H-NMR (Bruker 300 Ultrashield). The hydrolysis of one cyclic acetal produces one acetone molecule and the hydrolysis of one acyclic acetal produces one acetone and one methanol molecule. Therefore, from the relative ratio of the peaks from acetone, methanol, and the anomeric carbon on dextan, the cyclic:acyclic ratio of acetal substitution and degrees of substitution per 100 glucose could be determined [18]. A further description of NMR analysis and examples of NMR spectra of degraded Ac-DEX may be found in the Supporting Information by Bachelder et al. [55] A figure depicting a typical NMR output is given in Figure 9.

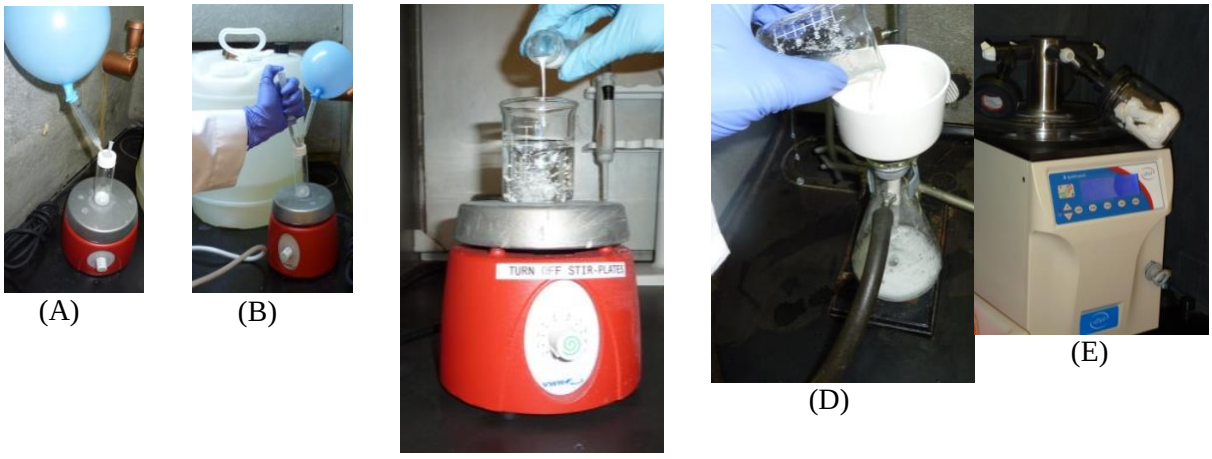


Figure 8: Fabrication of Ac-DEX.
(A) Reactants (Dextran, p-toluenesulfonate, and 2-methoxy-propene) are prepared in a nitrogen purged glass vial. (B) Reactants are dissolved in DMSO under anhydrous conditions. Reactants are reacted for a fixed time as per desired polymer degradation characteristics. Reaction is quenched with triethylamine. (C) Polymer is precipitated in basic water. (D) Polymer is isolated by filtration. (E) Polymer is lypophilzed and stored at -20° C.

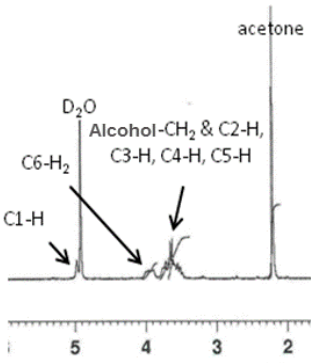


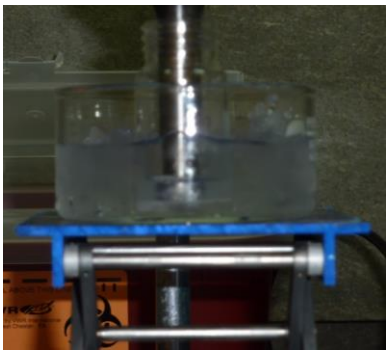
Figure 9: NMR Spectra for Ac-DEX

NMR structure of Ac-DEX is fabricated and the degraded polymer is analyzed via NMR to determine the cyclic coverage and degradation rate. For Resiquimod particles a cyclic coverage of approximately 44% is used, which results in a degradation of approximately 3 hours for 71K MW polymer.

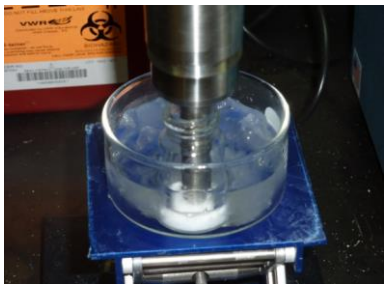
3. Fabrication of Ac-DEX Microparticles

Nanoparticles are classified by the NIH as particles smaller than 100 nms. For passive targeting of phagocytes, a diameter larger than 200 nm is desired. As we have a semi-polydisperse mixture of particles, that are mainly micro-sized to passively target DCs, we will refer to our particles as microparticles, abbreviated as nanoparticles (np). Microparticles containing resiquimod and proteins isolated from bioterrorism agents (*B. anthracis, or F. tularensis*) were prepared using a double emulsion (w/o/w) evaporation method similar to the technique described by Bachelder *et al*[33] (Figure 10).

(A)



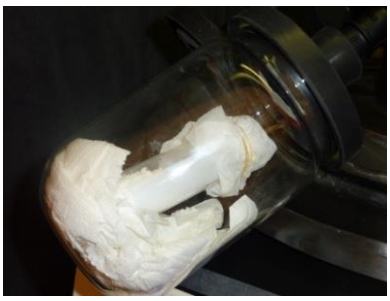
(B)



(C)

(D)

(E)



(F)

Figure 10: Fabrication of Ac-DEX Micro-/Micro-particles
(A) An two phase emulsion is prepared of Ac-DEX in dichloromethane (DCM) and 3% poly(vinyl alcohol) (PVA) in PBS. (B) Emulsion is placed in a water bath with sonicator tip inserted at emulsion interface. (C) The emulsion is sonicated forming a white frothy solution of nanosized particles. (D) The emulsion solution is added to a 0.3% PVA solution to allow for solvent (DCM) to evaporate. (E) The solvent is left to evaporate in while the continuous phase is stirred (3 hrs). Isolation through centrifugation. (F) Microparticles are lypophilzed and stored at -20° C.

3.1 Ac-DEX Particle Formation via Sonication

To synthesize sonicated microparticles, Ac-DEX (100 mg) and resiquimod (3 mg) were dissolved in DCM (1 mL) and added to 3% PVA. The mixture was vortexed for 30 seconds, sonicated for 30 seconds (Misonix Ultrasonic Liquid Processor), and the formed emulsion was immediately pipetted into a spinning solution of 0.3% PVA. The reaction mixture was allowed to spin for two hours to evaporate the solvent and allow for particle hardening. To recover the microparticles, each formulation was subjected to centrifugation (5 min, 20,000x g, 4 °C). The supernatant was discarded, and the resulting microparticle sediment was washed with basic water to remove excess drug and PVA (2 x 5 min, 20,000 x g, 4 °C). The microparticles were then suspended in basic water and lyophilized for two days to yield resiquimod-loaded Ac-DEX microparticles. To produce blank microparticles, the same procedure was followed, except no resiquimod was added.

3.2 Ac-DEX Particle Formation via Homogenization

Homogenized microparticles were prepared via a double-emulsion technique (water/oil/water) [56] (Figure 11). To fabricate the homogenized microparticles, protein or lysate in PBS (200 µL) was added to Ac-DEX (100 mg) dissolved in dichloromethane (DCM, 2 mL) and homogenized for 30 seconds (Polytron PT 10-35 Homogenizer, 20,500 RPM). This first emulsion was then added to 3% poly(vinyl alcohol) (PVA) in PBS (17 mL). The resultant mixture was homogenized for 30 seconds (Polytron PT 10-35 Homogenizer, 20,500 RPM) and the emulsion was immediately poured into a spinning solution of 0.3% PVA (40 mL). The same washing procedure was performed as described for the sonicated microparticles.

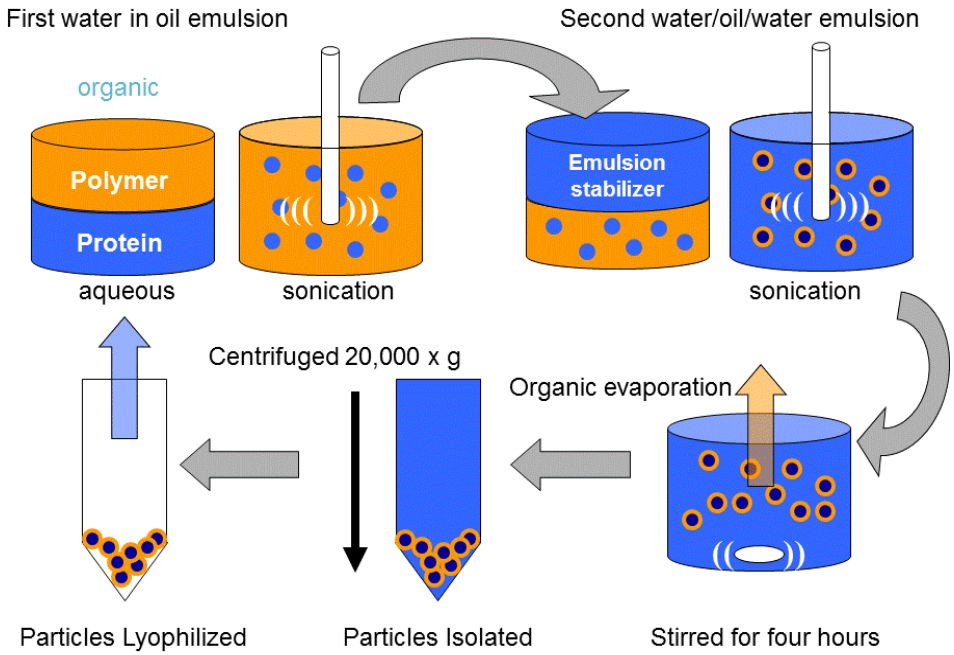


Figure 11: Schematic of Double Emulsion Process

Schematic of formation of Ac-DEX nanoparticles through emulsion chemistry. Ac-DEX and drug are dissolved in the organic layer (dichloromethane – DCM) with a protein layer of PBS and protein or lysate. This is homogenized and added to a water based layer that contains the stabilizer polyvinyl alcohol (PVA). The immiscible layers are then homogenized. DCM is then evaporated over time and the particles isolated through centrifugation. After washing, the particles are freeze dried (lyophilized) and stored at 0°C.

4. In vitro evaluation of Resiquimod Ac-DEX Microparticles

4.1 SEM Visualization of Resiquimod Microparticles

Microparticles were suspended in basic water at 10 mg/mL, and a small amount (20µL) was placed on a SEM (aluminum) pin stub (Ted Pella; Redding, CA). The samples were allowed to air dry, and then sputter coated with a layer of palladium/gold alloy for 120 seconds. The samples were imaged with a FEI NOVA NanoSEM 400 (Figure 12).

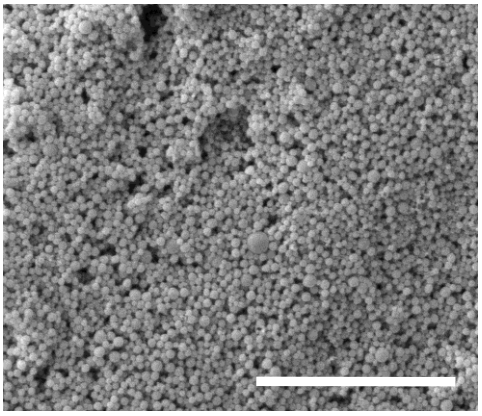


Figure 12: Resiquimod microparticles for in vitro analysis

Scanning electron micrograph of representative Ac-DEX particles encapsulating resiquimod. Scale bar represents 10 microns.

4.2 Determination of Resiquimod Loading

Ac-DEX microparticles containing resiquimod were suspended in triplicate in 0.3M sodium acetate buffer (pH 5). Aliquots were taken and analyzed for drug content by measuring the fluorescence of resiquimod via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). Blank Ac-DEX particles were analyzed in a similar fashion and used to determine background fluorescence. The experimental resiquimod concentration in each particle was determined by comparison with a standard curve of resiquimod. The encapsulation efficiency was determined by the equation: EE (%) = 100 * (experimental resiquimod concentration) / (theoretical resiquimod concentration). Also, the resiquimod percent weight loading (w/w) was determined by the equation: weight loading (%) = 100 * (loaded resiquimod in mg) / (amount of polymer in mg). The values for the original preparation of resiquimod particles, for in vitro analysis are given in Table 3.

%Encapsulation	Wt loading (mg/100mg Ac-DEX)
9.6	0.29

Table 3: Encapsulation efficiency and weight loading of resiquimod microparticles
Encapsulation efficiency and weight loading of Ac-DEX microparticles encapsulating resiquimod, with an initial loading of 3 mg drug/100 mg Ac-DEX.

4.3 Cellular Analysis of Resiquimod particles

4.3.1 Cell Study Preparation

Macrophages (RAW 264.7; ATCC, Manassas, VA) were grown and maintained as per the manufacturer's guidelines. To make the cell media, 50 mL of fetal bovine serum (Hyclone, Pittsburgh, PA) and 5 mL of penicillin-streptomycin (Fischer, Pittsburgh, PA) were added to 500 mL of Dulbecco's Modified Eagle's Medium (Fischer, Pittsburgh, PA). Cells were maintained at 100% relative humidity, 37°C, and 5% CO_2 for the duration of the experiments.

Macrophages were plated at concentration of 1 x 10^4 cells/mL and incubated for 24 hours in two 96-well plates. After 24 hours, the media in each well was replaced with media containing resiquimod - loaded Ac-DEX microparticles, blank Ac-DEX microparticles, or free resiquimod at the same concentrations, all in triplicate. The media used in one plate was spiked with lipopolysaccharide (LPS) (100 ng/mL) to promote nitric oxide (NO) production, whereas the media used in the other plate contained no LPS to act as a control. The cells were incubated for another 24 hours at these conditions and nitric oxide production was assessed.

4.3.2 Nitrite Analysis

A Griess assay was performed to determine the nitric oxide production by the macrophages. The supernatants from each well were removed and centrifuged (15,000 x *g*, 4 °C, 10 min), and 50 µL of the resulting supernatant was withdrawn. The Griess reagents (Promega, Fitchburg, WI) were added as per the manufacturer's instructions, and standard nitrite concentrations were prepared. The absorbance was measured at 540 nm and compared with the standard curve to determine nitrite concentration and subsequent nitric oxide production. The NO concentrations for the microparticles and free drug were standardized with respect to 0 µM. The results of the Griess assay are given in Figure 13.

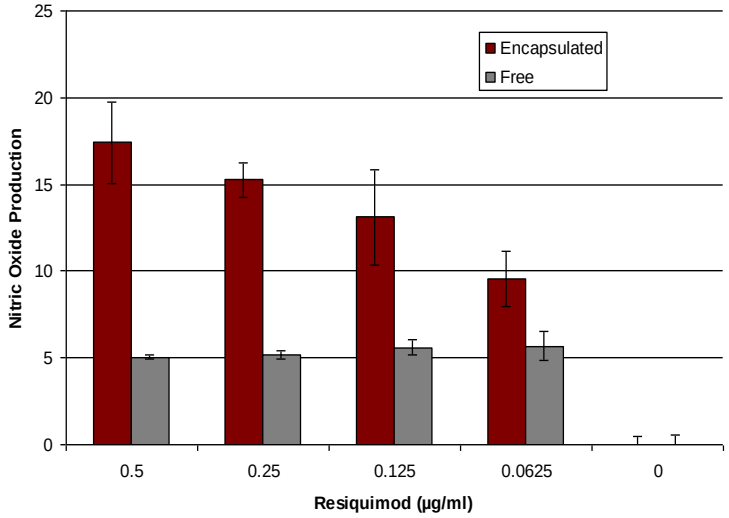


Figure 13: Nitric oxide production of RAW macrophages cultured with Ac-DEX microparticles encapsulating resiquimod

Resiquimod was loaded at 4 mg/100 mg of Ac-DEX and an encapsulation efficiency of 7% was achieved (0.28 mg resiquimod/100 mg of Ac-DEX microparticles). Macrophages were seeded at $1x10^5$ cells/mL into a 96-well plate and cultured for 24 hours. Unencapsulated (free) and encapsulated resiquimod was then added at the indicated concentrations. Nitric oxide production was then evaluated after 24 hours with a Greiss assay. Data is presented as average ± standard deviation. Nitric oxide production is in units of µg/mL. n=3.

5. Evaluation of Known Pathogen #1: Anthrax

5.1 Introduction

B. anthracis (Anthrax) is a spore-forming bacterium, which potentially can lead to lethal disease in humans and other mammals (primarily herbivores). Additionally, *B. anthracis* is one of the few bacteria that have actually been used as a bioweapon[57]. Upon initial exposure to macrophages, *B. anthracis* rapidly geminates and begins the production of its virulence factors and capsule. This leads to a rapid dissemination throughout the host and eventual septicemia [58]. The principal virulence factor of *B. anthracis* is a multi-component toxin secreted by the pathogen that consists of three separate gene products designated protective antigen (PA), lethal factor (LF) and edema factor (EF). These components act in concert with each other to induce deleterious effects on the host. PA is the central molecule in the development of toxicity as cleaved PA binds to human capillary morphogenesis protein 2 or tumor endothelial marker 8 where it provides binding sites for EF and LF[59,60]. These complexes are then taken up by a clatherin dependent mechanism where changes in pH cause pore formation and entry of EF and LF into the host[61,62]. Protection against anthrax is associated with a humoral antibody immune response directed against PA and possibly EF and LF[63]. The major immunogenic stimulants from *B. anthracis* are contained in two large plasmids. pOX1 contains the information for the production of anthrax toxins including EF, LF and PA. pOX2 is responsible for carrying the information necessary to create the capsule and is the missing component in the attenuated Sterne strain of Anthrax [64].Although the capsule also contributes to anthrax pathogenesis in mice, it is not clear if this is true in higher mammals such as non-human primates and rabbits.

We plan on using recombinant PA as the antigenic target for immunization against *B. anthracis* Sterne strain. Previous work has shown [65,66] that the injection of recombinant PA, in conjunction with adjuvants increase the titer of antibodies against *B. anthracis* and is capable of inducing protection of the animal against subsequent infection. In addition, we have access to copious amounts of PA allowing for the facile testing of the immune response against PA. Simple ELISA type assays can be easily used to quantify the immune response against a model bioterrorism agent. Finally, we chose to immunize against *B. anthracis* Sterne strain because there is high demand on improving the current vaccine. *B. anthracis* vaccine is administered in a 6-dose series over 18 months and requires yearly boosters. Due to this complicated immunization schedule the Institute of Medicine has called for development of a new anthrax vaccine[67].

We recently have obtained preliminary data showing that the encapsulation of resiquimod in Ac-DEX particles drastically enhances the activity of resiquimod in macrophages and dendritic cells. We chose to use resiquimod, since TLR7 and TLR8 expression in both murine and humans is conserved. Unlike other stimulants such as CpG, resiquimod can activate a wide range of monocyte populations that exist in both human and murine populations. In addition, resiquimod is a highly organic soluble molecule that allows for the facile encapsulation of an immunostimulant in a microparticle without the need of complicated incorporation techniques.

We hypothesize that the encapsulation of resiquimod and PA in the same microparticle will drastically enhance the immune response against PA. The enhancement in both humoral and cellular responses will result in a protective immune response against subsequent challenge by *B. anthracis* Sterne strain.

5.2 Experimental Conditions

For all challenge and live fire evaluations, we will have up to nine experimental conditions that are listed in Table 4.

Name	Abbreviation	Protein (ug/mouse)	Resiquimod (ug/mouse)	Alum (mg)
Phosphate Buffer Solution (sham)	PBS	0	0	0
Blank microparticles	NP	0	0	0
Unencapsulated protein	Protein	15	0	0
Unencapsulated protein with Alum (traditional formulation)	Protein + Alum	15	0	2
Encapsulated Resiquimod	Resiquimod/NP	0	8	0
Free Protein and Encapsulated Resiquimod	Protein + Resiq/NP	15	8	0
Encapsulated Protein	Protein/NP	15	0	0
Encapsulated Protein and in separate microparticles, encapsulated Resiquimod	Protein/NP + Resiq/NP	15	8	0
Encapsulated Resiquimod, Free Protein and alum	Resiq/NP + Protein + Alum	15	8	2

Table 4: Experimental Groups for all in vivo studies
The experimental groups, abbreviations, protein amount, resiquimod amount and alum concentrations are given.

5.3 Humoral Response to Protective Antigen

5.3.1 Introduction

The generation of a humoral response is very important for an effective vaccine. The incorporation of peptides and proteins in microparticles has been shown to increase the level of antigen specific antibodies compared to free antigen, and antigen injected with complete Freund's adjuvant (CFA) [68,69]. We tested the efficacy of Ac-DEX particles in generating a humoral response against an encapsulated protein. We vaccinated mice subcutaneously (n=10 per group) on day 0 and day 7 with the experimental groups presented in Table 4. Antibody class and titer were measured by ELISA. Splenocytes isolated from vaccinated mice were also evaluated for cytokine production in response to antigen (i.e. antigen recall assay).

5.3.2 Particle Preparation

Ac-DEX particles were used to generate a humoral response against *B. anthracis* Sterne strain. Ac-DEX was used to encapsulate the recombinant protein (Figure 14), protective antigen (PA) from *B. anthracis*, known to provide protection against a subsequent infection from *B. anthracis*. A comparison was performed measuring the difference in efficacy between free PA, and PA encapsulated in Ac-DEX. We chose using *B. anthracis* Sterne strain because it is a relatively well characterized bioterrorism agent that has defined antigens that when immunized against will provide protection against. We have access to these antigens, allowing the encapsulation of antigens in Ac-DEX particles. Using these recombinant proteins as antigens has the advantage of allowing a quantitative measurement of the humoral response generated against anthrax. Resiquimod particles were also fabricated for this experiment Figure 15.

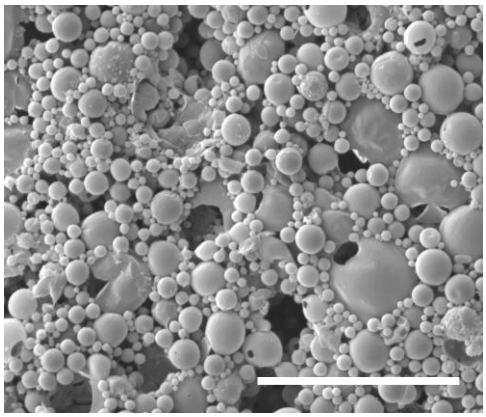


Figure 14: Ac-DEX particles encapsulating protective antigen for evaluation of humoral response
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating protective antigen. Scale bar is 10 microns.

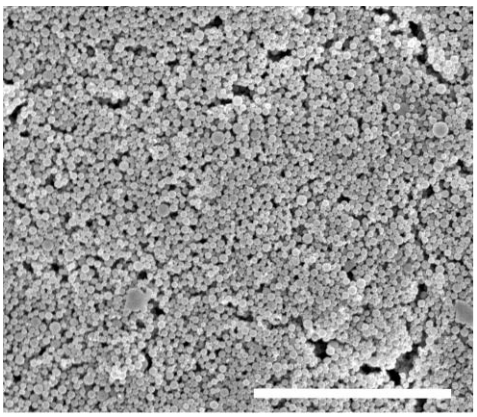


Figure 15: Ac-DEX microparticles encapsulating resiquimod for evaluation of humoral response
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod, as used for non-challenge in vivo experiment. Scale bar represents 10 microns.

5.3.2.1 Fluorescamine Assay for Determination of Protein Encapsulation

A fluorescamine assay was used to determine the protein content of the microparticles. Ac-DEX microparticles containing PA were suspended in triplicate in 0.3M sodium acetate buffer (pH 5). Aliquots were taken and analyzed for protein content by adding 50 uL of fluorescamine solution (3 mg fluorescamine/mL in acetone) and measuring its fluorescence at 400 nm/460 nm (excitation/emission) via a plate reader (FlexStation 3 Benchtop Multi-Mode Microplate Reader). Blank Ac-DEX particles were analyzed in a similar fashion and used to determine background fluorescence. A standard curve was prepared using the same protective antigen used for encapsulation. The encapsulation of resiquimod was determined as outlined in 4.2.

	%Encapsulation	Wt loading (mg/100mg Ac-DEX)
Resiquimod	7.0	0.21
Protective Antigen	92	1.38

Table 5: Particle loadings for non-challenge in vivo experiment
Ac-DEX microparticle loadings for resiquimod and protective antigen. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and protective antigen 1.5 mg protein/100 mg Ac-DEX.

5.3.3 Animal Vaccination

Studying the humoral response was the second major milestone in this grant. Ac-DEX particles were used to induce a humoral response in mice against the PA of *B. anthracis* Sterne strain. The desired metric for this experiment is that the mice were injected subcutaneously with experimental conditions listed in Table 4 as performed as outlined in Figure 16. Antibody class and titer were measured by ELISA. An antigen recall assay was performed on isolated splenocytes and cytokine production was evaluated with a pro-inflammatory panel in luminex.

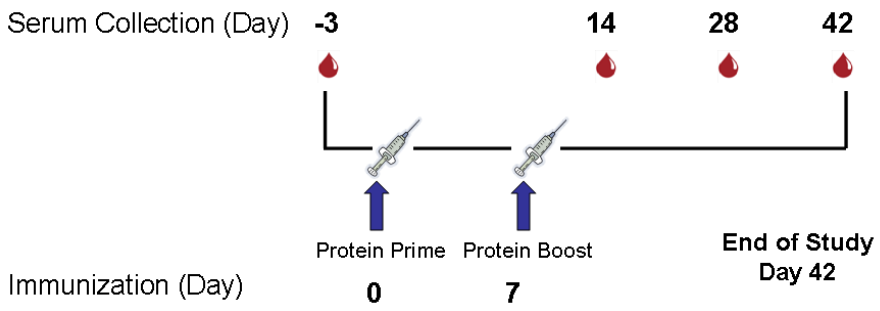


Figure 16: Schedule for Humoral Response Evaluation
A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via sub-cutaneous injection. The numbers indicate the study day in which the indicated procedure was performed.

5.3.3.1 Observed Lumps at Site of Vaccination

A/J mice were vaccinated at 0 and 7 days. Observable lumps developed in the vaccination region (the flank) after the second vaccination. These lumps primarily developed with groups Protein/NP + Resiq/NP and Resiq/NP + Protein + Alum. A photograph of a represented lump is given in Figure 17.

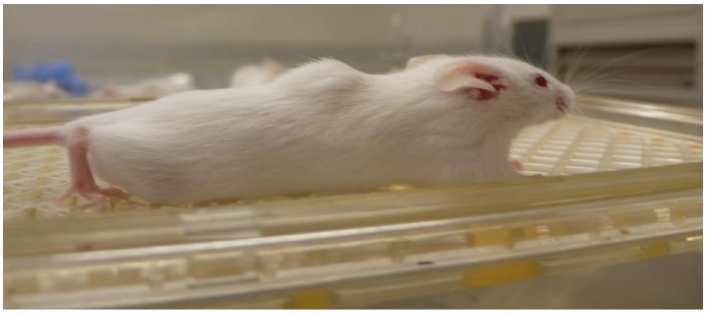


Figure 17: Micrograph of vaccination lump developed at vaccination site
Experimental conditions were injected in the nape of the neck of the animal. This is what was observed after two vaccinations. Lumps were observed in all groups vaccinated with encapsulated resiquimod.

5.3.4 Cytokine Analysis

5.3.4.1 Supernatant Isolation

An antigen recall assay specific for protective antigen was performed as outline in Figure 18. Spleens isolated from vaccinated mice on day 42. The organs were homogenized and seeded in well plates. Exogenous PA was added to the plates and incubated for 72 hours. Supernatants were isolated from the culture via centrifugation.

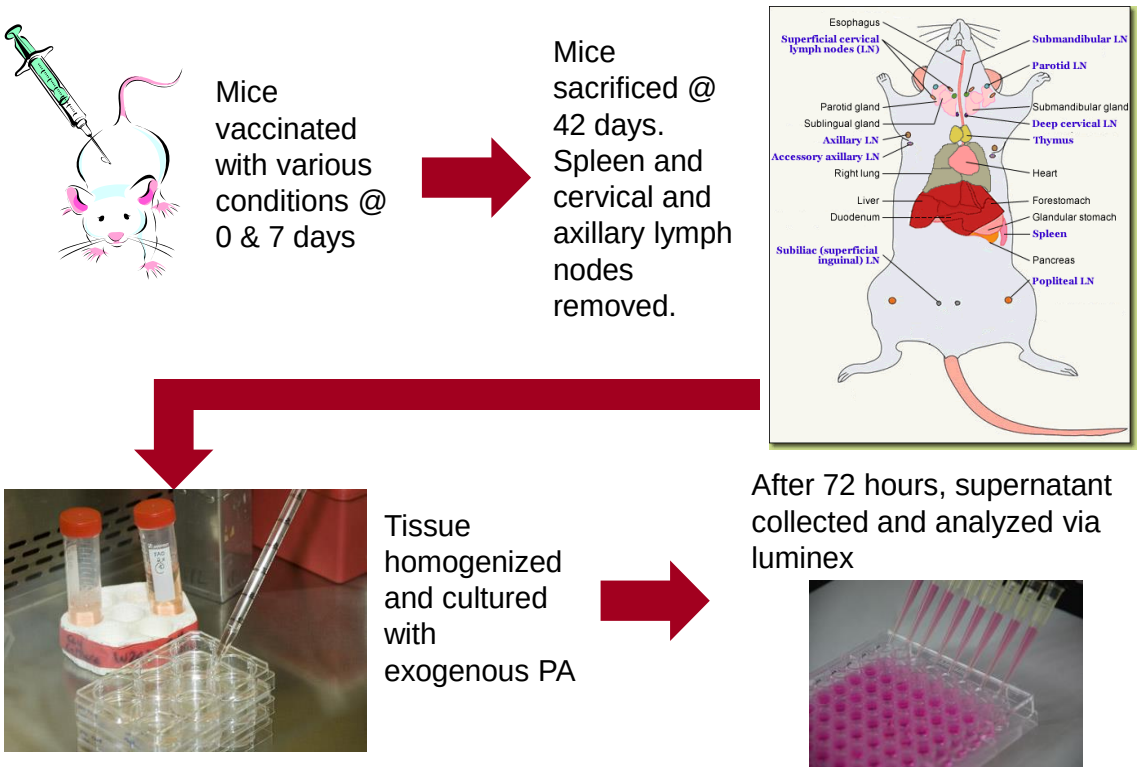


Figure 18: Schematic of how supernatants for cytokine analysis were collected
Spleens form vaccinated animals were isolated, homogenized and cultured with exogenous protective antigen (PA). After 72 hours, the cell supernatant was collected and analyzed for cytokine production.

5.3.4.2 Luminex Procedure

Beads of defined spectral properties are conjugated to protein-specific capture antibodies and added along with samples (including standards of known protein concentration, control samples, and test samples), into the wells of a filter-bottom microplate. Target protein binds to the capture antibodies over the course of a 2-hr incubation. After washing the beads, protein-specific, biotinylated detector antibodies are added and incubated with the beads for 1 hr. Next, excess biotinylated detector antibodies are removed, and streptavidin-conjugated fluorescent protein, R-Phycoerythrin (SAV-RPE), is added and incubated for 30 min. SAV-RPE binds to the biotinylated detector antibodies, forming a four-member, solid-phase sandwich. After washing to remove unbound SAV-RPE, the beads are analyzed with a Luminex® detection system. By monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin (RPE) fluorescence, the concentration of one or more proteins can be determined. A schematic of this process is given in Figure 19.

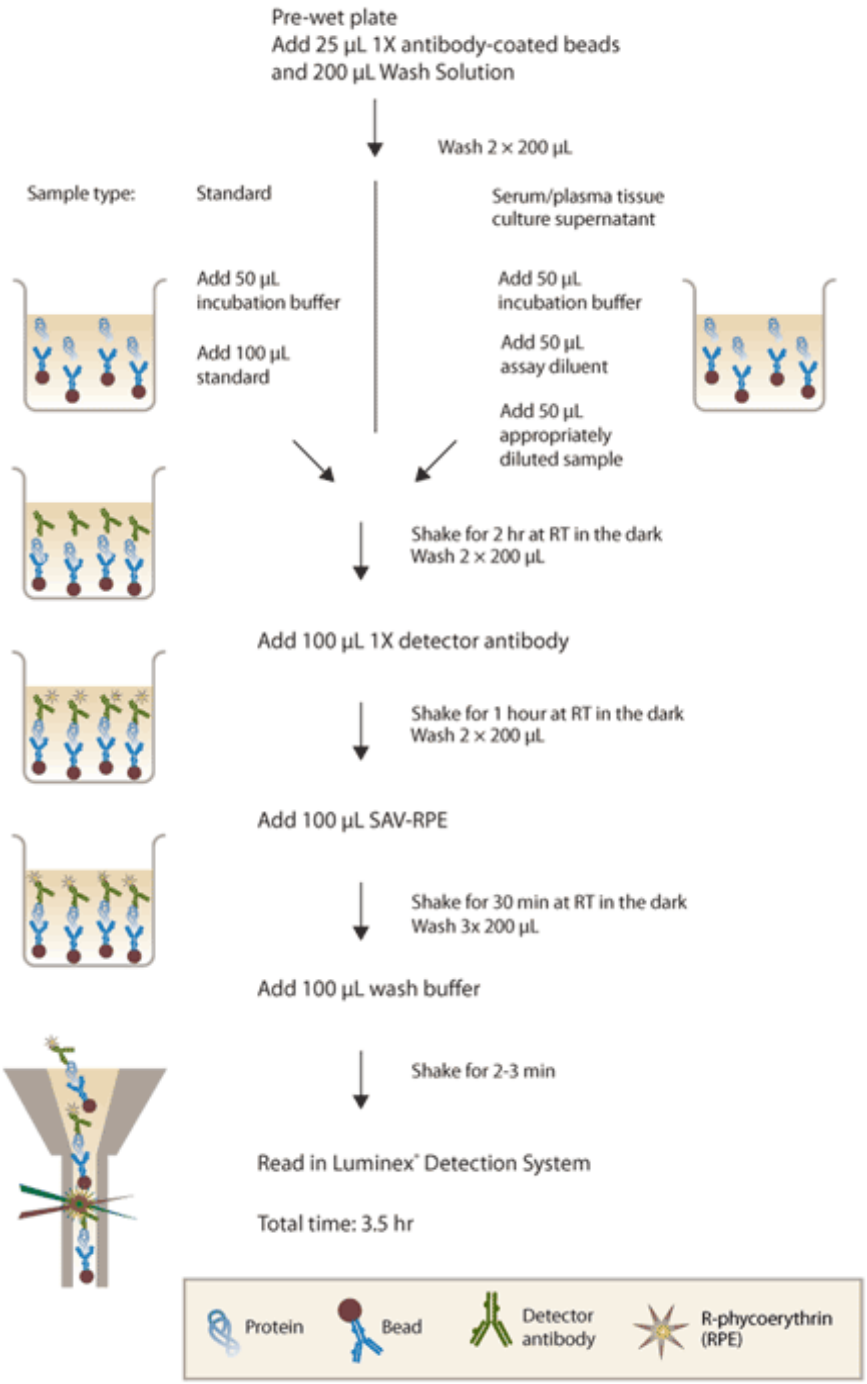


Figure 19: Luminex assay protocol

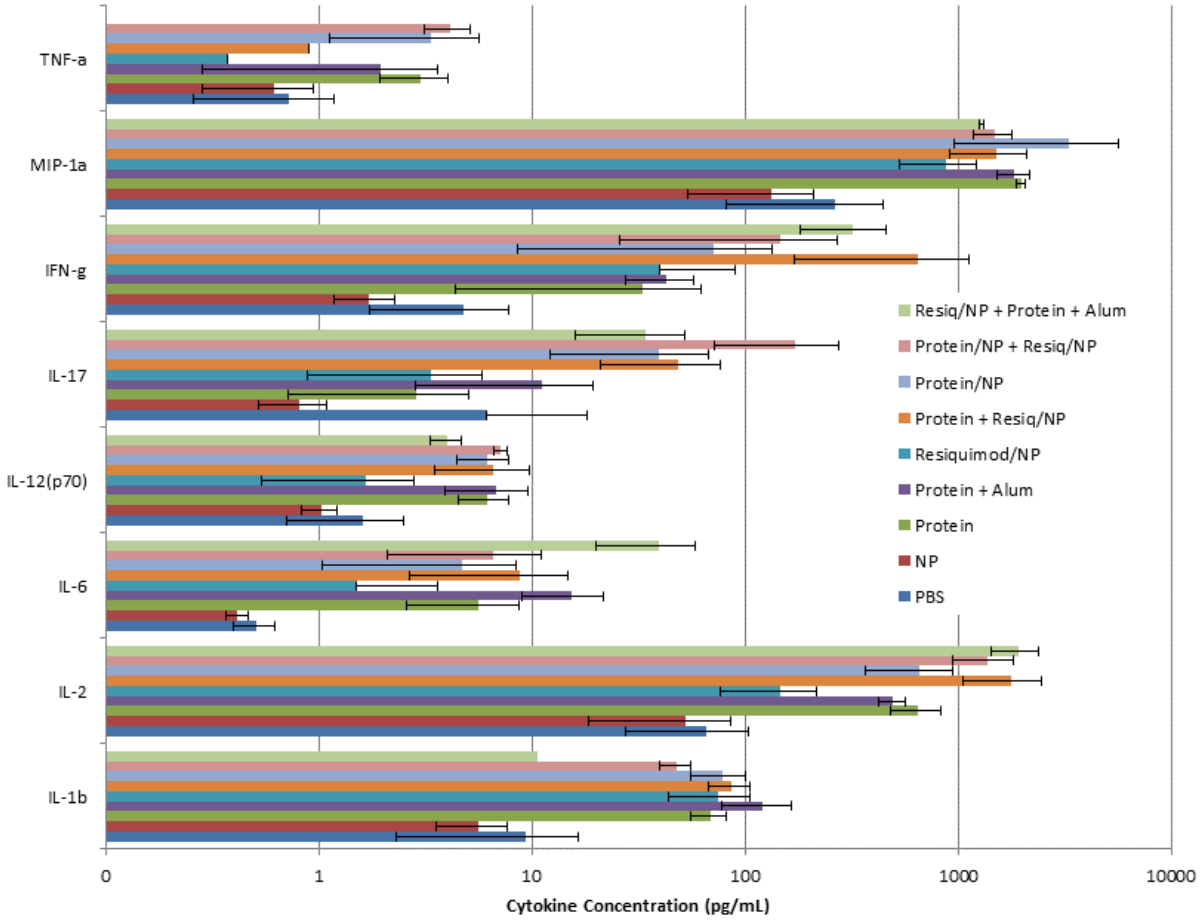


Figure 20: Cytokine production for protective antigen recall assay
Cytokine production for protective antigen recall assay with splenocytes isolated from mice vaccinated at day 0 and 7 with indicated experimental conditions (Table 4). Encapsulated condition is indicated with /NP. Resiq indicates 8 µg/mouse of resiquimod. Protein is protective antigen at 15 µg/mouse. Data is presented as average ± standard deviation. n=0-5.

5.3.5 Protective Antigen Specific Antibody Titer

The generation of a humoral response is very important for an effective vaccine. We vaccinated A/J mice subcutaneously (n=10 per group) on day 0 and day 7 with experimental groups (Table 4). Antibody class and titer were measured by ELISA on day -3, 14, 28, and 14. Antigen specific antibody titer was determined by absorbing PA onto ELISA plates. An indirect ELISA was then performed wherein blood samples at serial dilutions were added to the plate and incubated. This incubation step allowed the PA specific antibodies to bind to the protein. After a washing step, a secondary anti-IgG antibody with congregated horse-radish peroxidase (HRP) was added and incubated. Another washing step was performed and HRP activity was measured with a reactive substrate. Substrate concentration was measured with a plate reader and antibody concentration was determined by comparison to a standard curve. Antibody titer for PA vaccination is given in Figure 21. For antibody isotypes, IgG1 and IgG2 secondary antibodies were used. Isotypes are presented in Figure 22. An informative table contrasting the difference between the two isotypes is given in Table 6.

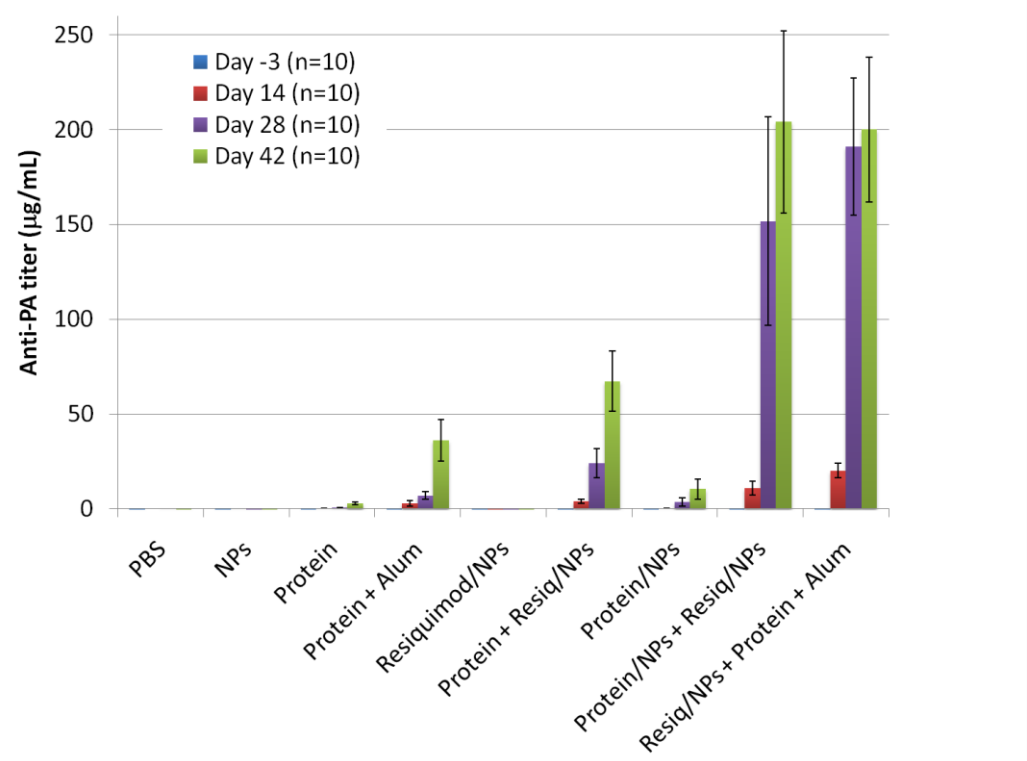


Figure 21: Protective antigen specific antibody titers
Protective Antigen specific antibody titers for blood samples taken from mice vaccinated on day 0 and 7 with indicated experimental condition (Table 4). Data is presented as average ± standard deviation.

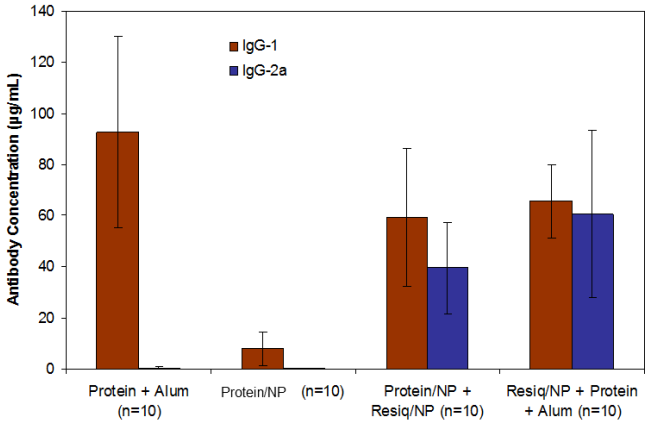


Figure 22: IgG isotype antibody concentration
Antibody isotype concentrations for anthrax vaccination studies for select experimental conditions (Table 4). Data is presented as average ± SEM.

	IgG_1	IgG_2
Molecular mass (kD)	146	146
Susceptibility to proteolytic enzymes	Moderate	Low

Number of allotypes	4	1
Adult serum level range (g/l) (mean, g/l)	4.9-11.4 (6.98)	1.5-6.4 (3.8)
Proportion of total IgG (%)	43-75	16-48
Half-life (days)	21	21
Antibody response to proteins	High	Moderate
CD4 Response	T_H2	T_H1

Table 6: Informative table contrasting IgG1 and IgG2 antibodies

5.3.6 Toxin Neutralization Assay

5.3.6.1 Introduction

A toxin neutralization assay (TNA) was performed to help evaluate the effectiveness of the antibodies generated with vaccination in the prevention of anthrax. A TNA is performed to evaluate in vitro the viability of cells with the addition of lethal factor and PA. The addition of these two anthrax proteins would kill. Antibodies that are effective at preventing the biding of protective antigen from binding to the cell or other components of anthrax (e.g. lethal factor) would reduce cell death and therefore be effective antibodies in the lysis of cells due to anthrax infection. The procedure for a TNA is given in Figure 23.

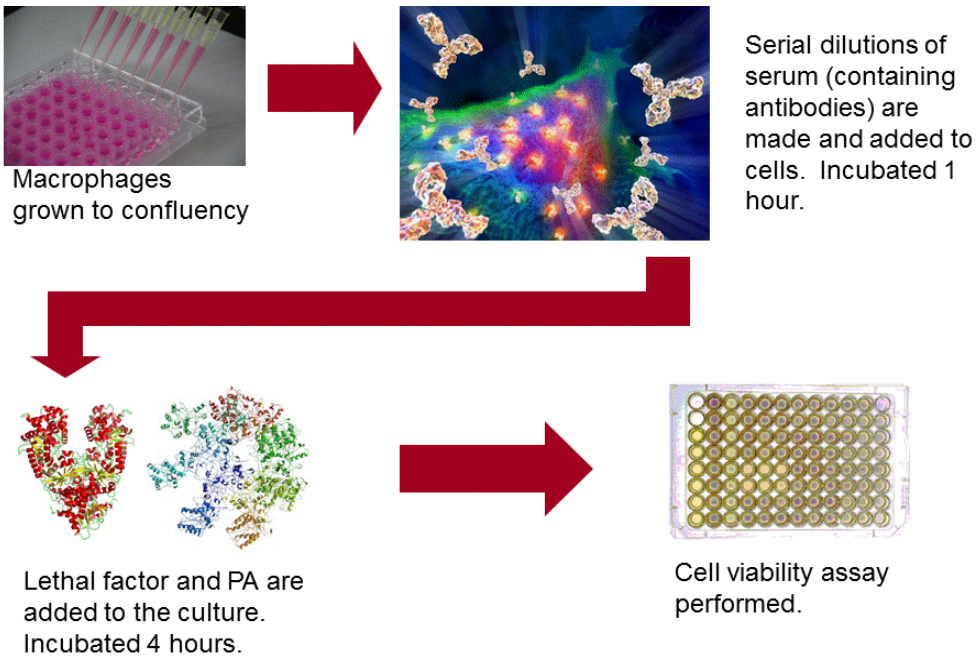


Figure 23: Schematic of how anthrax toxin neutralization assay is performed

5.3.6.2 TNA Results

To evaluate the effectiveness of the protective antigen and lethal factor used in the TNA, a standard curve needs to be created that indicates the concentration of PA that results in 50% of cell death at a fixed concentration of lethal factor. This standard curve is given in Figure 24.

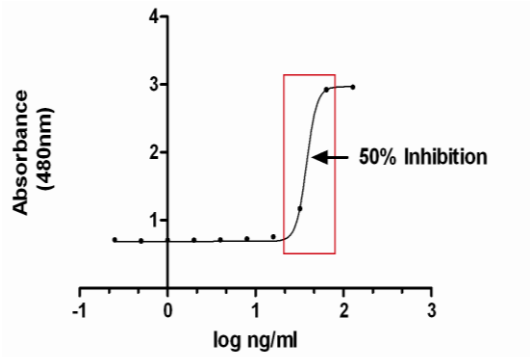


Figure 24: Standard curve for toxin neutralization
Standard curve indicating the concentration of protective antigen results in 50% inhibition of cell death due to anthrax.

Figure 25 presents the data for Neutralizing Equivalence Units (NEU) for blood samples isolated from vaccinated mice on day 14, 30 and 45. One NEU corresponds to the amount of antibody needed to neutralize one anthrax antitoxin.

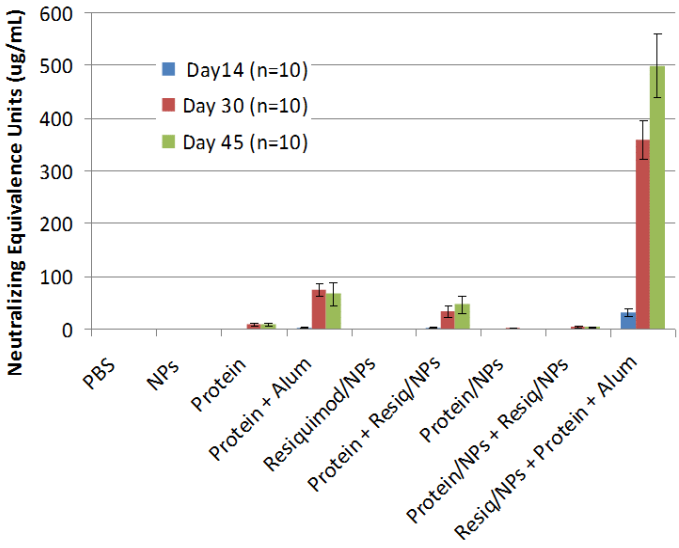


Figure 25: Neutralizing equivalence of blood samples
Neutralizing Equivalence Units (NEU) of anti-PA antibodies in blood samples for the indicated experimental conditions (Table 4). A higher NEU generally corresponds to better protection against anthrax infection. Data is presented as average ± standard deviation.

5.3.6.3 Comparison of TNA to Other Vaccination Methods

Figure 26 presents data that compares the toxin neutralization activity of other vaccination methods for anthrax.

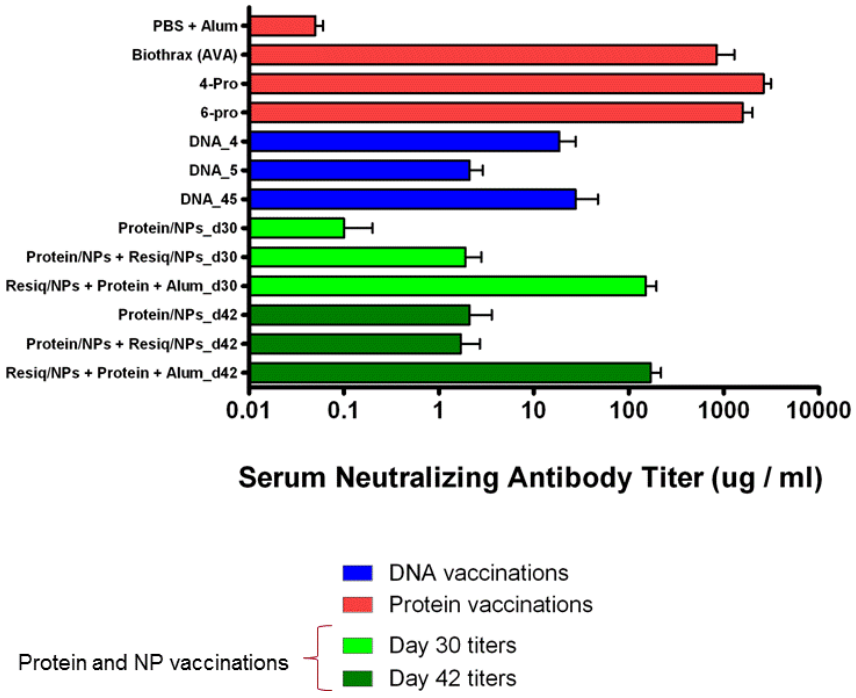


Figure 26: Comparison of TNA data to other vaccination methods
The concentration of serum neutralizing antibodies is for our experimental conditions (Table 4) are compared to the concentration of neutralizing antibodies gained through alternative experimental conditions, including Biothrax, the vaccine currently given for prevention of anthrax. For the microparticle experiments, d42 represents samples taken at day 42.

5.4 Anthrax Challenge: Low dose

5.4.1 Introduction

We tested the efficacy of Ac-DEX particles in generating a protective immune response after three subsequent challenges from *B. anthracis* Sterne strain, one week apart. Prior to challenge, mice were vaccinated subcutaneously (n=10 per group) on day 0 and day 7 with experimental conditions (Table 3). After immunization mice were challenged with a lethal dose of either *B. anthracis* Sterne strain on day 14, 21, and 28. Mice were euthanized when they become moribund as reflected by a hunched posture, ruffled fur and immobility. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by a log-rank test. A p-value<0.10 were considered significant. If lethal challenge results in a death rate much lower than the desired rate (>5%), then the amount of protein that is injected were increased and the experiment were repeated.

5.4.2 Particle Preparation

Ac-DEX polymer and particles were fabricated and characterized as indicated in sections 2 and 3. Particles encapsulating protective antigen, resiquimod or nothing (blanks) were fabricated. Representative scanning electron micrographs are presented in Figure 27 and Figure 28 for protective antigen and resiquimod, respectively. The loadings for these particles are presented in Table 9.

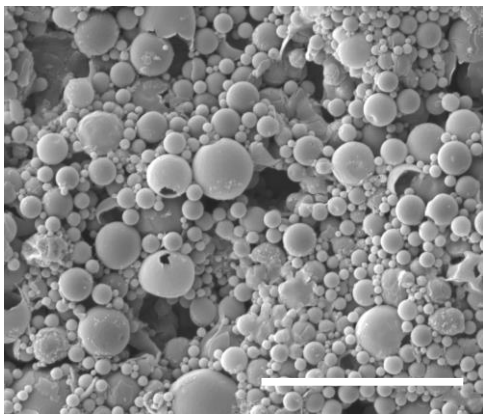


Figure 27: Ac-DEX particles encapsulating protective antigen for low-dose experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating protective antigen. Scale bar is 10 microns.

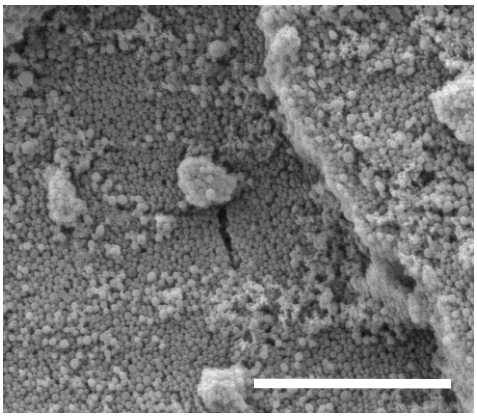


Figure 28: Ac-DEX microparticles encapsulating resiquimod for low-dose experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod, as used for cytokine and antibody non-challenge experiments. Scale bar represents 10 microns.

	%Encapsulation	Wt loading (mg/100mg Ac-DEX)
Resiquimod	9.6	0.29
Protective Antigen	84.4	1.27

Table 7: Particle loadings for low-dose experiment
Ac-DEX microparticle loadings for resiquimod and protective antigen. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and protective antigen 1.5 mg protein/100 mg Ac-DEX.

5.4.3 Animal Challenge

A/J mice (n=10) were challenged with a low-dose ($4.3x10^4$ CFU on average) as per the schedule outlined in Figure 29.

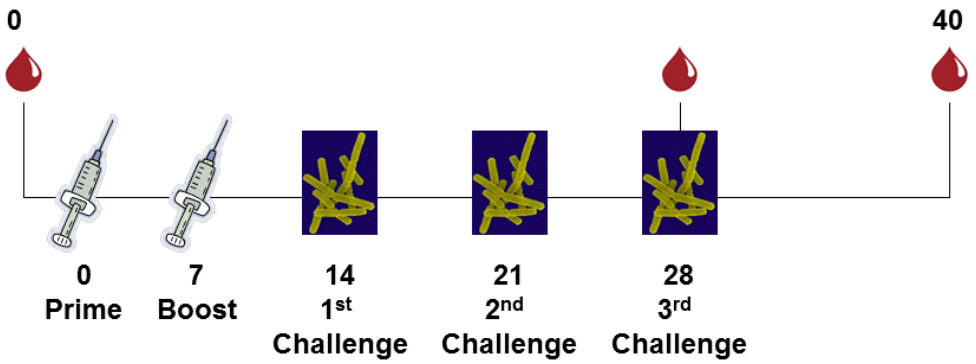


Figure 29: Schedule for low-dose study

Schedule of Anthrax vaccination challenge. A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via sub-cutaneous injection. The numbers indicate the study day in which the indicated procedure was performed. Animals were given an intra-tracheal.

5.4.3.1 Observed Lump Ruptures at Vaccination Sites

Similar to what was observed with humoral studies, a lump developed at the vaccination site in mice after the second vaccination (day 7). However in contrast to the humoral studies, many of the lumps opened up and turned into abscesses (Figure 30). The frequencies of these accesses are noted in Table 8.

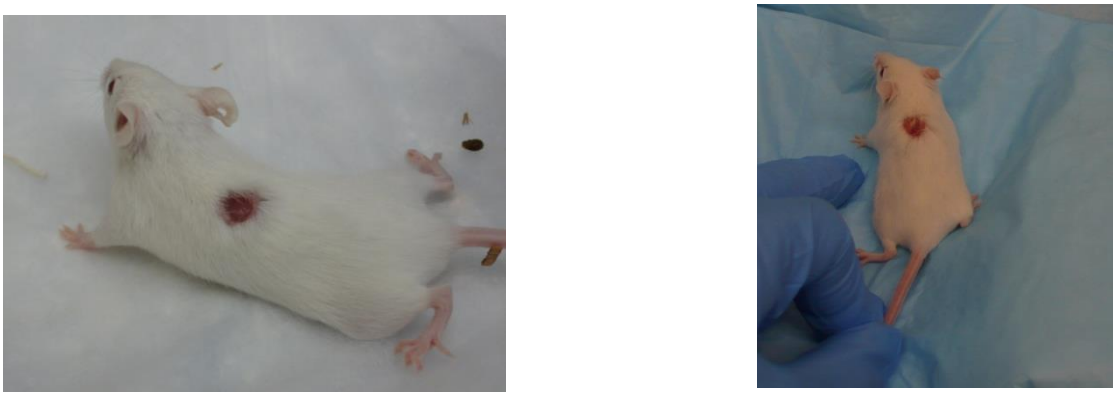


Figure 30: Rupture abscesses in vaccinated mice

As with the in vivo non-challenge experiments, all animals vaccinated with encapsulated resiquimod developed lumps after two vaccinations. However, several mice also developed abscesses at the site of inoculation within one week after the second vaccination.

Vaccination	Frequency of abscesses
Alum + PA + Resiq/NP	15/15
Resiq/NP + PA/NP	4/15

Table 8: Frequency of abscesses

Frequency of abscesses in groups that developed abscesses in vaccinated/challenge groups. Other groups did not develop abscesses.

5.4.3.2 Challenge Experiment

A/J mice were vaccinated and challenged as outlined in Figure 29. As anthrax progressed in the control mice, signs of *B. anthracis* infection began to become pronounced, including edema in the chest,

and face (Figure 31 & Figure 32). These observances were primarily in the PBS or blank experimental groups and were observed shortly before animal death.

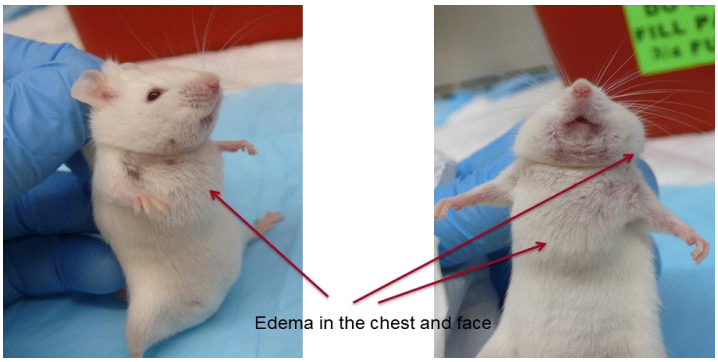


Figure 31: Edema of the chest and face
Example of edema in the chest and face of mice challenged with anthrax from the PBS group. Arrows highlight regions of edema.

Figure 32: Edema of the face
Example of edema in the face of mice challenged with anthrax from the blank microparticle group. Arrows highlight regions of edema.

The survival of the vaccinated animals for the experimental groups were observed and reported in Figure 33.

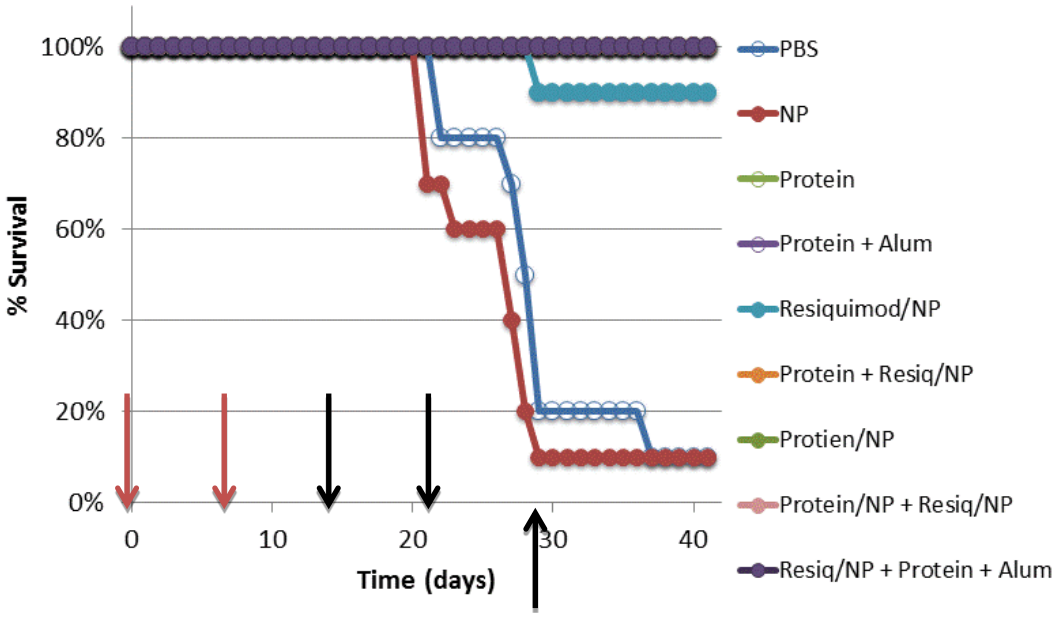


Figure 33: Survival of mice in low-dose experiment

Survival curves of mice given a low dose of anthrax. The red arrows represent sub-cutaneous vaccination with indicated experimental conditions and the black arrows indicate intra-tracheal challenge (average 4.3 x 10^4 CFUs). Experimental groups protein, protein + alum, protein + resiq/NP, protein/NP, Protein/NP + Resiq/NP and Resiq/NP + Protein + Alum all report 100% survival and are overlapping on the graph.

Post mortem, either due to anthrax infection or sacrifice at day 42, mouse hearts were isolated, opened, and swabbed for bacteria. The swab was then streaked on agar plates containing 5% sheep's blood and CFUs were evaluated after 72 hours at 37°C.

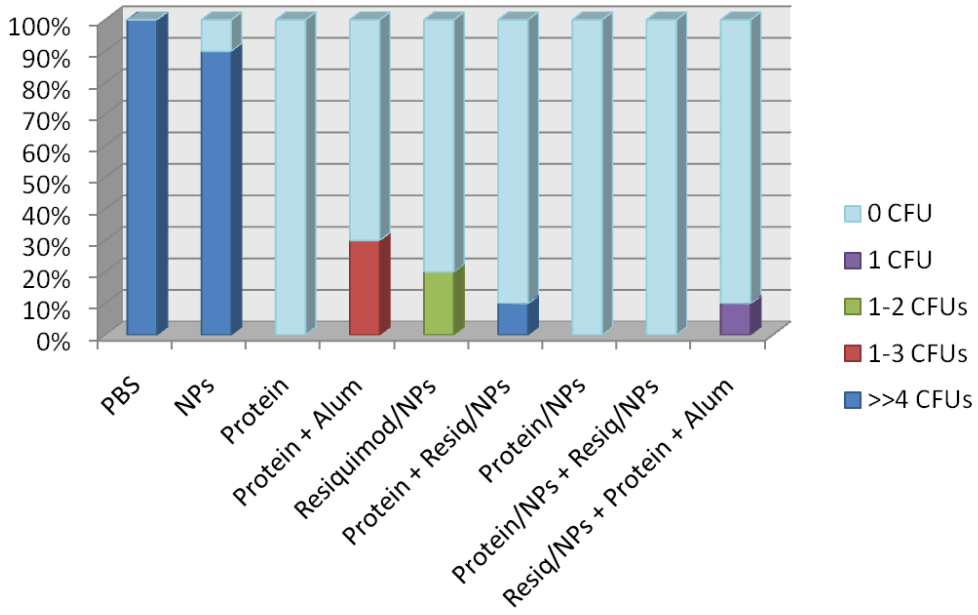


Figure 34: Organ bacterial loads in low-dose experiment

Bacterial loads in the heart for animals vaccinated and challenged with a low-dose of anthrax. CFU = colony forming units. n=10.

5.5 Anthrax Challenge High Dose

5.5.1 Introduction

Animals vaccinated and challenged with a low dose of anthrax (section 5.4) did not develop edema or perish at a rate expected for such a bacterial challenge. For that reason, a group of A/J mice set aside for further cytokine analysis (n=5) were challenged on day 18 with a higher dose of anthrax (1.66×10^5 CFUs) via intra-tracheal challenge.

5.5.1 Particle Preparation

Protective antigen and resiquimod particles were fabricated for a high dose anthrax challenge. Scanning electron micrographs of the particles are given in Figure 35 and Figure 36. The encapsulation efficiency of the particles is given in Table 10.

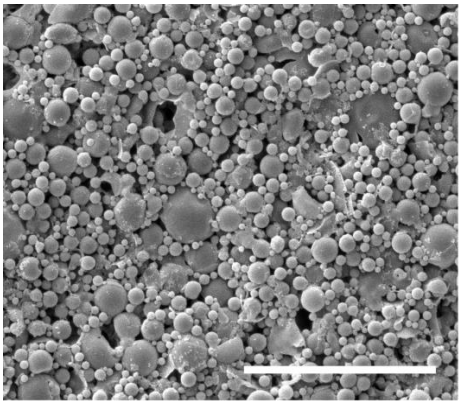


Figure 35: Ac-DEX particles encapsulating protective antigen for high-dose experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating protective antigen. Scale bar is 10 microns.

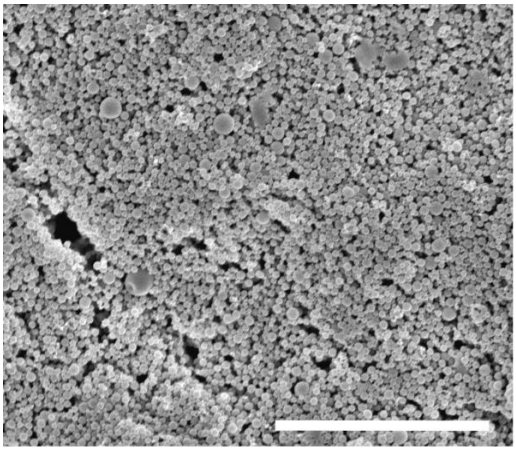


Figure 36: Ac-DEX microparticles encapsulating resiquimod for high-dose experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod, as used for high-dose experiment. Scale bar represents 10 microns.

	%Encapsulation	Wt loading (mg/100mg Ac-DEX)
Resiquimod	8.7	0.26
Protective Antigen	99.3	1.49

Table 9: Particle loadings for high-dose experiment

Ac-DEX microparticle loadings for resiquimod and protective antigen. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and protective antigen 1.5 mg protein/100 mg Ac-DEX.

5.5.3 Animal Challenge

A/J mice (n=5) were vaccinated and challenged as outlined in the Figure 37 with the groups that produced significant levels of antibodies (Figure 21) (i.e. Protein + Alum, Protein + Resiqu/NP, Protein/NP, Protein/NP + Resiqu/NP, and Resiqu/NP + Protein + Alum).

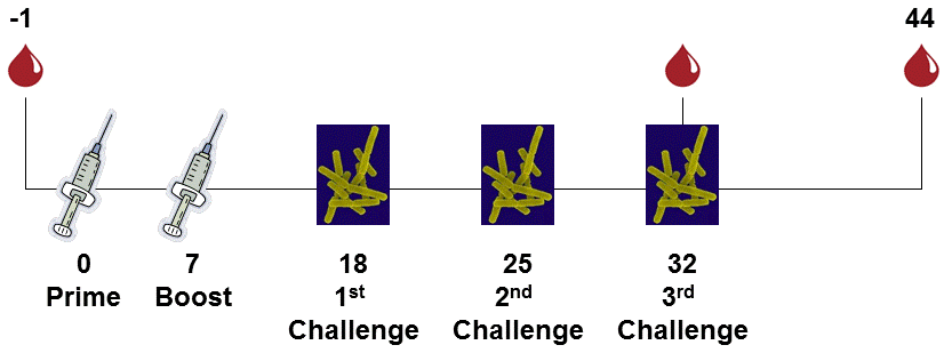


Figure 37: Schedule for high-dose study

Experimental outline for High dose anthrax challenge experiment. The numbers indicate the study day in which the indicated procedure was performed. A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via subcutaneous injection. Animals were given an intra-tracheal.

The survival for the high dose study is given in Figure 38.

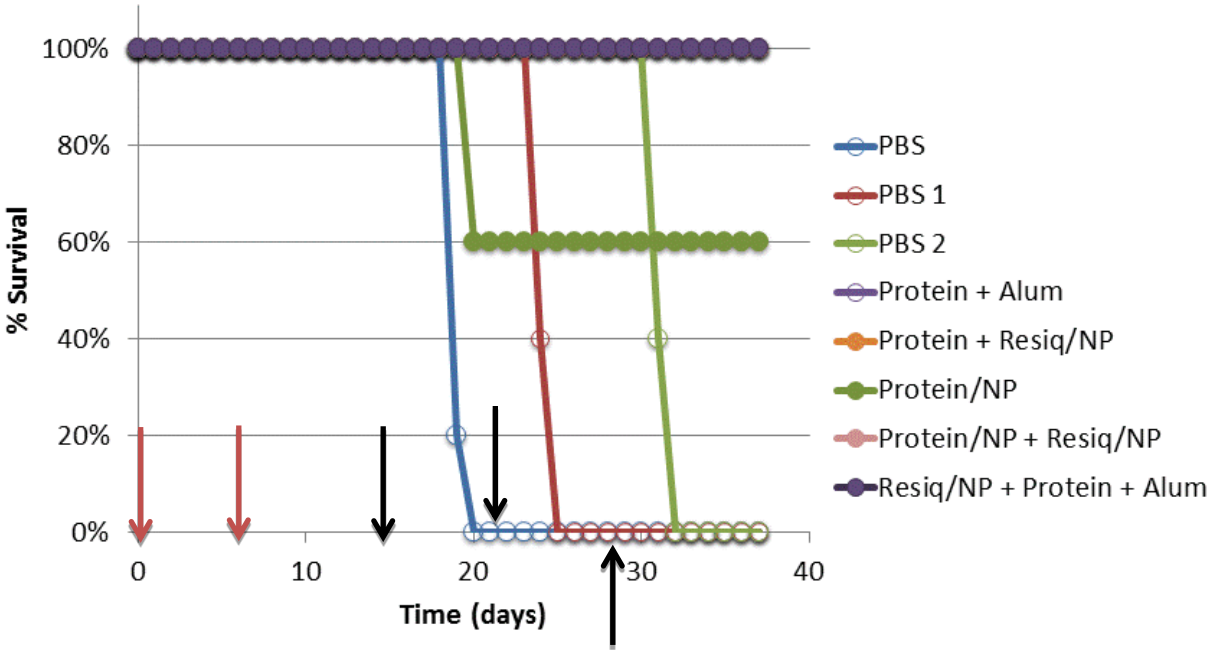


Figure 38: Survival of mice in high-dose experiment

Survival curves of mice given a high-dose of anthrax. The red arrows represent subcutaneous vaccination with indicated experimental conditions and the black arrows indicate intra-tracheal challenge (average

1.66 x 10^5 CFUs). Experimental groups protein + alum, protein + Resiq/NP, Protein/NP + Resiq/NP and Resiq/NP + Protein + Alum all report 100% survival and are overlapping on the graph.

Similar to the low-dose study, post mortem the hearts of the mice were swabbed and plated. CFUs were enumerated and are reported in Figure 39.

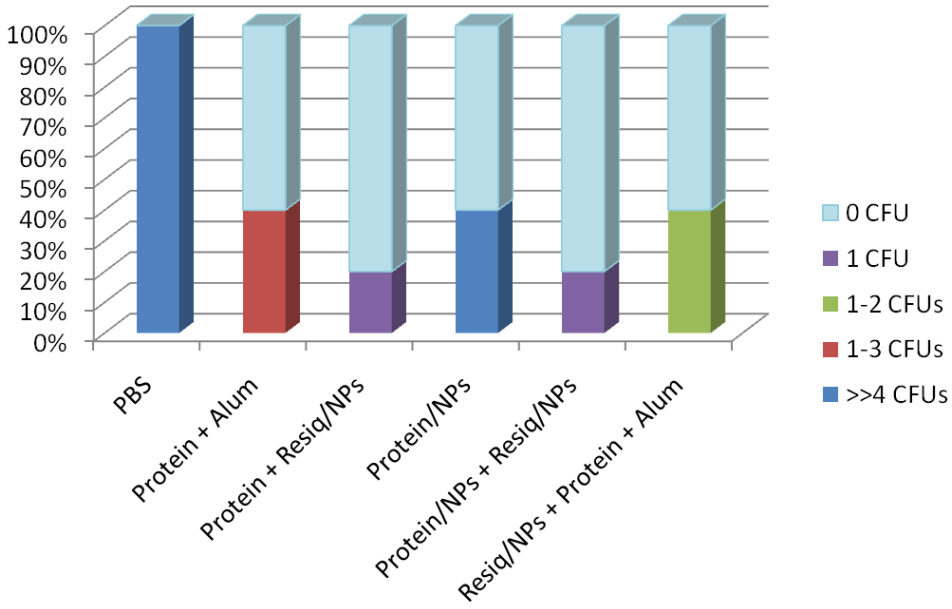


Figure 39: Organ bacterial loads in high-dose experiment
Bacterial loads in the heart for animals vaccinated and challenged with a high dose of anthrax. CFU = colony forming units. n=5.

5.6 Dose Sparing Anthrax Experiment

5.6.1 Introduction

In an attempt to delineate the survival curves reported for the low and high dose experiments, an experiment was performed wherein the antigen concentration delivered during vaccination was reduced. This dose sparing experiment was performed where antigen was delivered at a concentration of 15 µg/mouse and 5 µg/mouse. Previous experiments were performed at 15 ug/mouse.

5.6.2 Particle Preparation

Particles were prepared for the dose sparing experiment as outlined above. Scanning electron micrographs are reported for the prepared particles in Figure 40 and Figure 41. The encapsulation efficiency of the particles is reported in Table 10.

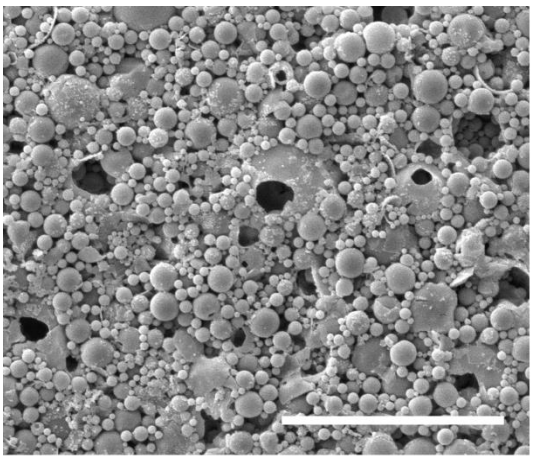


Figure 40: Ac-DEX particles encapsulating protective antigen for dose sparing experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating protective antigen. Scale bar is 10 microns.

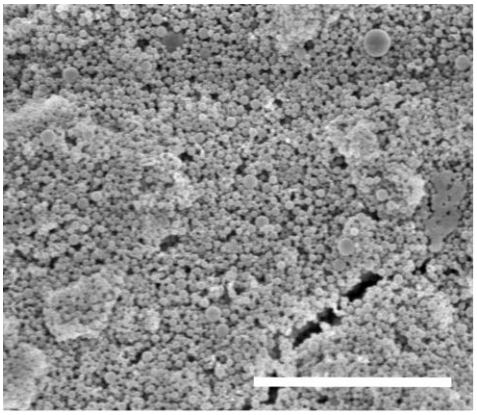


Figure 41: Ac-DEX microparticles encapsulating resiquimod for dose sparing experiment
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod, as used for high-dose experiment. Scale bar represents 10 microns.

	%Encapsulation	Wt loading (mg/100mg Ac-DEX)
Resiquimod	7.3	0.22
Protective Antigen	92.3	1.38

Table 10: Particle loadings for low-dose experiment
Ac-DEX microparticle loadings for resiquimod and protective antigen. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and protective antigen 1.5 mg protein/100 mg Ac-DEX.

5.6.3 Animal Challenge

For the dose sparing experiment, A/J mice (n=10) were vaccinated at challenged as outlined in Figure 42. The survival of the mice after challenge is reported in Figure 43.

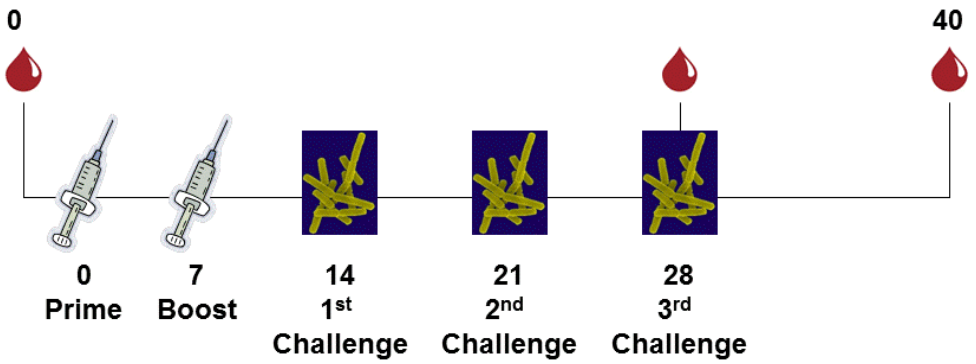


Figure 42: Schedule for dose sparing study

Schedule of Anthrax vaccination challenge. A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via sub-cutaneous injection. The numbers indicate the study day in which the indicated procedure was performed. Animals were given an intra-tracheal.

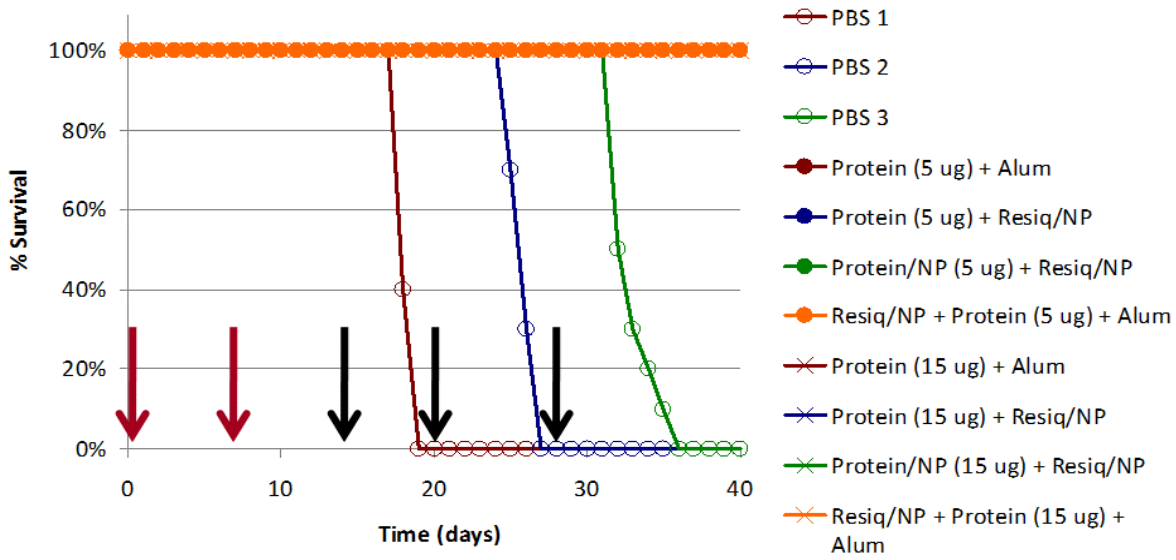


Figure 43: Survival of mice in dose sparing experiment

Survival curves of mice given different levels of protein and then challenged with anthrax. The red arrows represent subcutaneous vaccination with indicated experimental conditions and the black arrows indicate intra-tracheal challenge (average 9.5 x 10^5 CFUs). Experimental groups protein + alum, protein + Resiq/NP, Protein/NP + Resiq/NP and Resiq/NP + Protein + Alum all report 100% survival and are overlapping on the graph.

6. Evaluation of Known Pathogen #2: *F. novicida*

6.1 Introduction

F. tularensis an aerobic gram negative coccobacilli, is considered a class A bioterrorism agent due to its ease of spread, high mortality rates, low infectious doses, and potential social disruption [70]. There are four known species in the *Francisella* family: *tularensis*, *holarctica*, *medisiatica*, and *novicida*. *Tularenis* (Type A) is found primarily in North America, and is associated with rabbits and ticks while *holarctica* is typically associated with ticks and mosquitoes, and it produces reduced clinical phenotype with decreased infection and mortality rates [71].

Clinical presentation after exposure to *F. tularensis* varies. Incubation is between a few hours to 3 weeks and may ultimately present in one of two forms (Ulceroglandular or Typhoidal) [72,73]. Ulceroglanduar tularemia occurs in a majority of patients and resulting generic symptoms including fever, chills, headache, cough, myalgias, and painful lesions located around the site of vector transmission. Typhoidal tularemia occurs in about 25% of clinical cases but results in significant mortality. Clinical symptoms include hepatomegaly, splenomegaly, pneumonia, and high fever [72].

The host immune response to *F. tularensis* is poorly defined to date. Mice infected with the type A strain are unable to control the infection and quickly die, while mice infected with the type B strain are typically capable of managing the infection and clear the bacteria by day 30 post infection [74]. Alveolar macrophages are the dominant cell associated with host defense, although which macrophage associated receptor is responsible for inducing immune activation is still under debate [75]. Several studies have shown that even though the host is able to recognize *F. tularensis*, infection results in poor immune stimulation, and in fact actively induces immune suppression by reducing the host's ability to respond to PAMPS such as LPS [76].

In the following Experimental Aim, we present a method to vaccinate against *F. novicida*, a subspecies of *F. tularensis.* Since *F. tularensis* is a bioterrorism agent that does not have many epitopes characterized that provide protection against subsequent challenge, we will use it as a model unknown agent. The goal of this project is to develop a delivery vehicle that can provide universal protection against bioterrorism agents. We grew *F. tularensis*, lysed the bacteria, and incorporated the lysed proteins into our Ac-DEX particle system. Prior research has shown that the encapsulation of bacterial lysate in microparticles increases the protection of mice against a lethal challenge from a pathogen[77]. We hypothesize based on our preliminary data, that the encapsulation of resiquimod and bacterial lysate will drastically increase the immune response against lysate which will eventually provide protection against subsequent lethal challenges.

6.2 F. novicida LD_{50}

To evaluate the lethal dose of F. novicida, a LD50 study was performed where A/J mice were given intraperitoneal (i.p.) injections of various concentrations of bacteria and observed for survival. The schedule for the LD50 experiments is given in Figure 44. The results of the challenge are given in Figure 45.

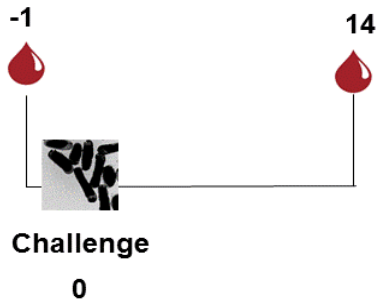


Figure 44: Schedule for LD50 study for F. novicida

A blood drop represents a collection of blood via lateral tail vein nick. Animals were challenged with i.p. injection.

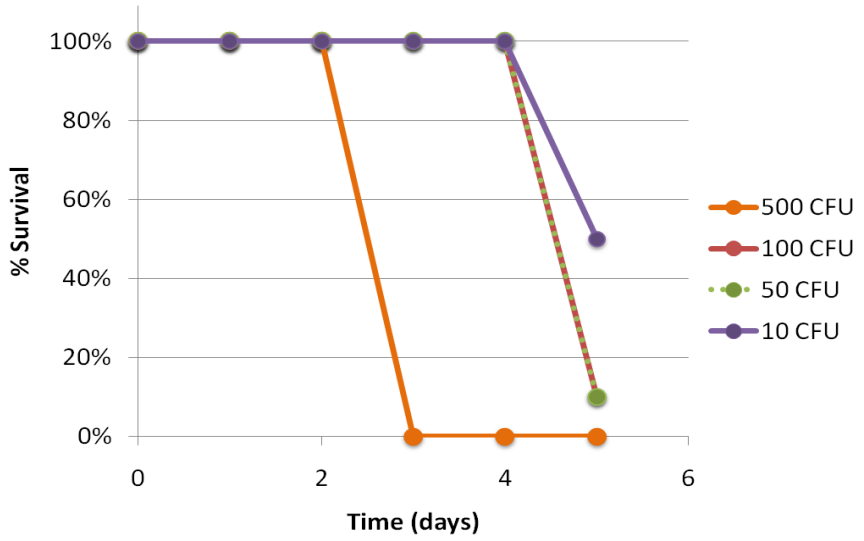


Figure 45: Survival of Mice in LD50 *F. novicida* study
LD50 challenge results for *F. novicida* lysate.

6.3 F. novicida Challenge

We present an innovative concept of encapsulating lysate from a bioterrorism agent in Ac-DEX particles. The encapsulation of lysate from a bioterrorism agent is innovative since prior knowledge of dominant epitopes that provide protection is not necessary. Proteins derived from the unknown agent can be encapsulated. We encapsulated the nontoxic fraction into Ac-DEX particles. Prior to challenge, mice were vaccinated subcutaneously (n=10 per group) on day 0 and day 7 with experimental conditions presented in Table 4. After immunization mice were challenged with a lethal dose of the unknown agent on day 14, 21, and 28. Anesthetized mice were challenged by intranasal administration of the unknown agent. Mice were euthanized when they become moribund as reflected by a hunched posture, ruffled fur and immobility. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by a log-rank test. A p-value<0.10 was considered significant. If lethal challenge results in a death rate much lower than the desired rate (>5%), then the amount of lysate that is injected was increased and the experiment was repeated.

6.3.1 Lysate Preparation

Lysate was prepared by growing up the bacteria, lysing it and isolating it through centrifugation (Figure 46). After isolation, the protein content was quantified and endotoxin was removed via Triton X-114 micellar removal (Figure 47). Endotoxin removal was performed in batches and for each batch the process was performed at least once. The final endotoxin concentration is reported in Table 11.

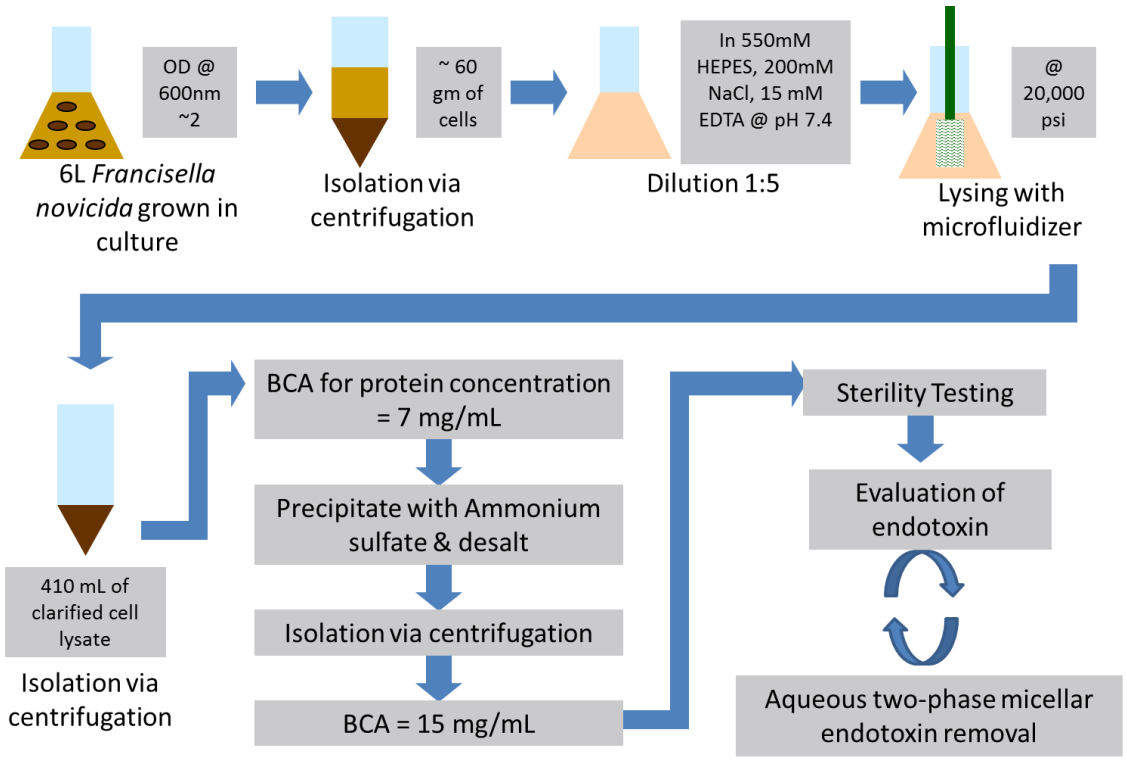


Figure 46: Preparation of *F. novicida* lysate

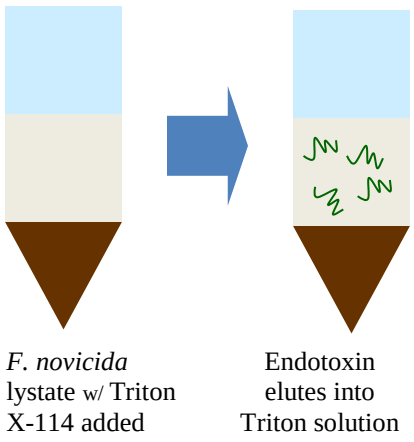


Figure 47: Schematic of endotoxin elution process

Run	Lysate (mg)	LPS (mg/mL)	LPS (EU/mg)
1	40	7.3	30.3
2	40.5	8.1	124
3	21	2.6	833
4	29.5	8.2	13.4
Pooled (-3)	**110**	**7.8**	**58.9**

Table 11: Endotoxin level of *F. novicida* lysate

6.3.2 Particle Fabrication

Ac-DEX particles were fabricated to encapsulate F. novicida lysate and resiquimod. Scanning electron micrographs of the particles are given in Figure 48 and Figure 49. To calculate the encapsulation efficiency of the lysate protein particles, a standard curve with lysate protein using the fluorescamine assay (5.3.2.1) was developed and used (Figure 50). The protein content of lysate was predetermined using BCA assay. The encapsulation efficiency of the particles is given in Table 12.

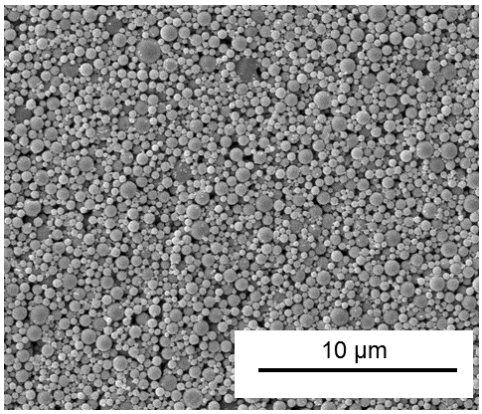


Figure 48: Ac-DEX microparticles encapsulating *F. novicida* lysate
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating *F. novicida* lysate. Scale bar is 10 microns.

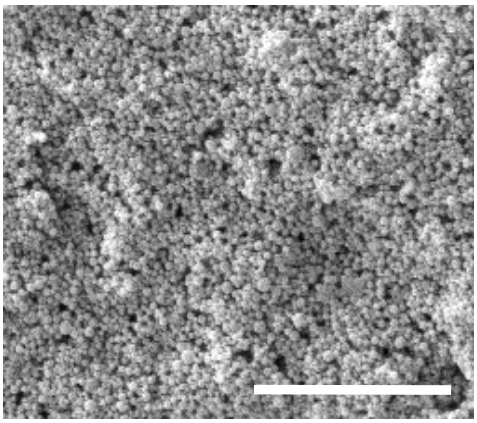


Figure 49: Ac-DEX microparticles encapsulating resiquimod for *F. novicida* experiments
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod. Scale bar is 10 microns.

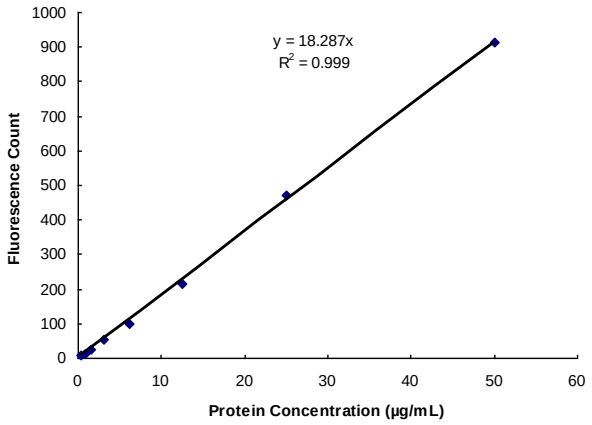


Figure 50: Standard curve for F. novicida lysate measurement
Standard curve of *F. novicida* lysate measured with fluorescamine assay. Fluorescamine assay detects lysine groups on proteins. The standard curve was used to calculate encapsulation efficiency.

Particle Set	Wt loading (mg/100mg Ac-DEX)	%Efficiency
***F. novicida* Lysate**	1.70 ± 0.14	47.3 ± 3.8
Resiquimod	0.22 ± 0.00037	7.4 ± 0.012

Table 12: Particle Loading for *F. novicida* Experiments
Encapsulation characteristics of Ac-DEX microparticles encapsulating *F. novicida* lysate. Ac-DEX microparticle loadings for resiquimod and protective antigen. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and *F. novicida* lysate protein 5 mg lysate/100 mg Ac-DEX.

6.3.3 Animal Vaccination

A/J mice were vaccinated and challenged (i.p. at an average 235 CFU of *F. novicida*) as per the schedule outlined in Figure 51.

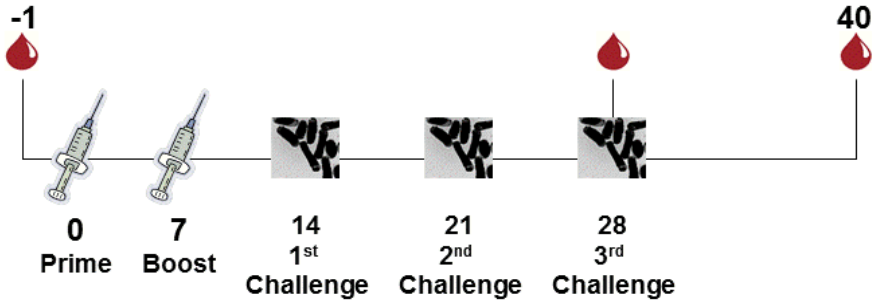


Figure 51: Schedule for *F. novicida* experiment
Experimental outline for *F. novicida* study challenge experiment. The numbers indicate the study day in which the indicated procedure was performed. A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via subcutaneous injection. Challenge is performed by i.p. injection.

6.3.3.1 Mouse Survival with Challenge

The survival of the mice were monitored and reported in Figure 52.

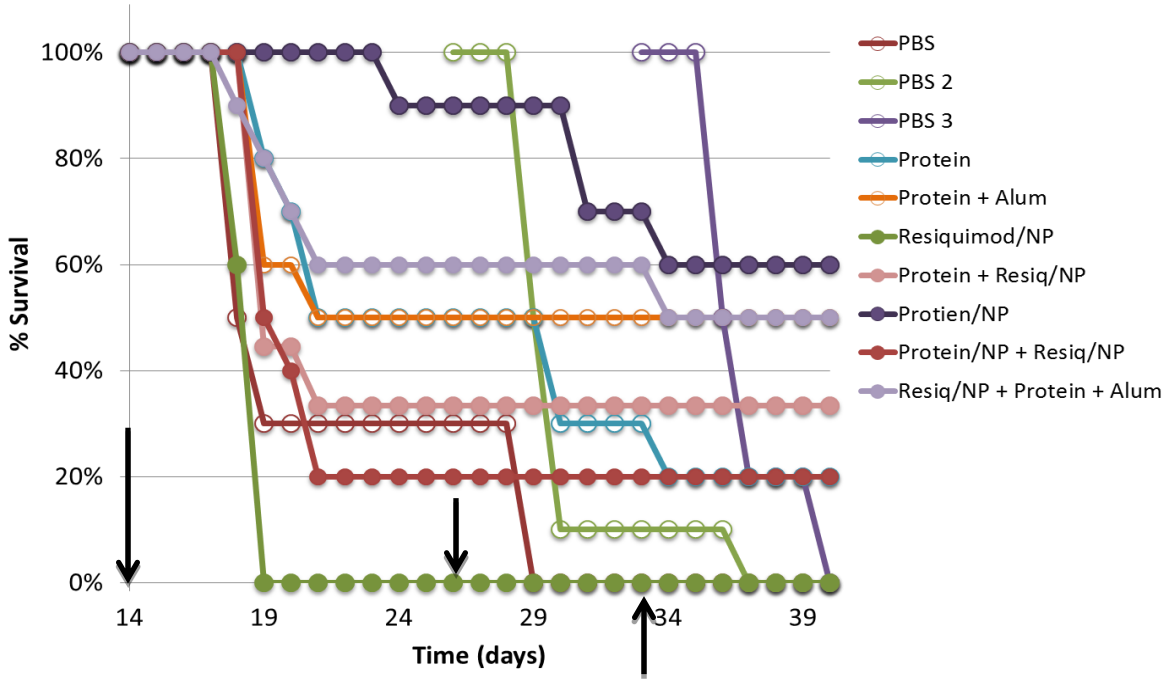


Figure 52: *F. novicida* survival results

Survival curves of mice given different levels Ac-DEX microparticles with lysate (Protein). Mice were vaccinated on day 0 and 7. The black arrows indicate i.p. challenge (average 235 CFUs).

6.3.3.2 Cytokine Analysis

Splenocytes isolated from the sacrificed animals were treated as indicated in Figure 18, except in place of protective antigen, *F. novicida* lysate was used. The results of the antigen recall assay are given in Figure 53.

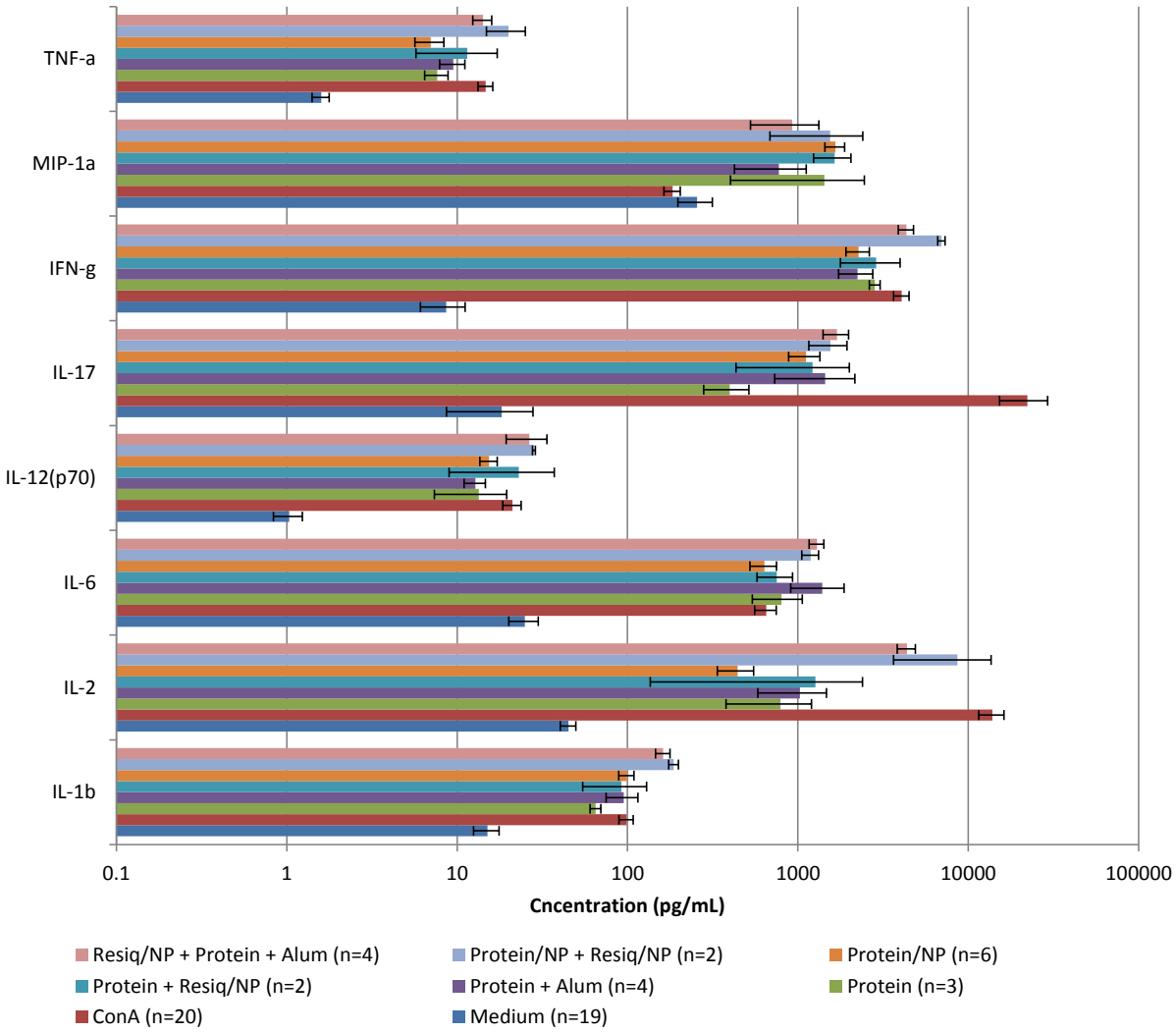


Figure 53: Cytokine production for *F. novicida* lysate recall assay
Cytokine production for *F. novicida* lysate recall assay with splenocytes isolated from mice vaccinated at day 0 and 7 with indicated experimental conditions (Table 4). Encapsulated condition is indicated with /NP. Resiq indicates 8 ug/mouse of resiquimod. Protein is *F. novicida* lysate at 20 µg/mouse. Data is presented as average ± standard deviation.

6.3.3.2 Lysate Specific Antibody Titer

In the same method outlined in 5.3.5, lysate specific antibody titer was measured from the isolated blood samples Figure 54. Antibody isotypes were also measured and reported in Figure 55. A breakdown of isotype concentration in each mouse is given in Figure 56 and Figure 57. This is the same data that is summarized in Figure 55.

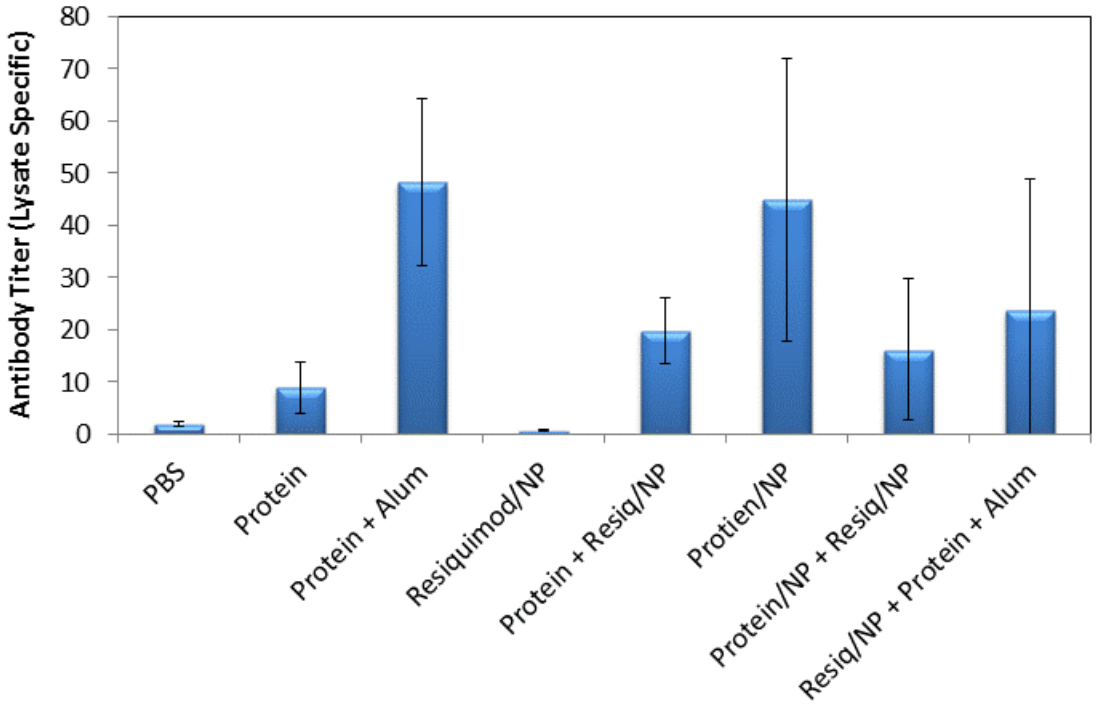


Figure 54: Day 14 *F. novicida* lysate antibody titer

F. novicida Lysate specific antibody titers for blood samples taken from mice vaccinated on day 0 and 7 with indicated experimental condition (Table 4). Data is presented as average ± standard deviation.

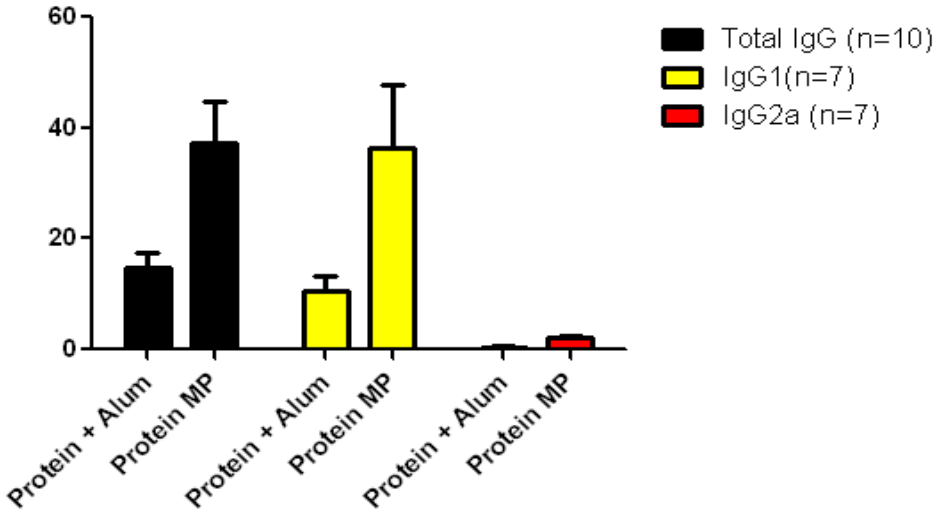


Figure 55: Day 14 *F. novicida* lysate antibody isotype

Plasma samples were isolated from mice after sub-Q vaccination on 0 and 7 days with either *F. novicida* lysate (Protein) + alum or an equal concentration of lysate encapsulated in Ac-DEX nano/mico-particles (particles). Mice were inoculated at 20 µg lysate /mouse. Mice were challenged day 21, 26 and 33 i.p with 232-402 times LD_{50} *F. novicida*. N represents the number of mice. Data is presented as an average +/- the standard error.

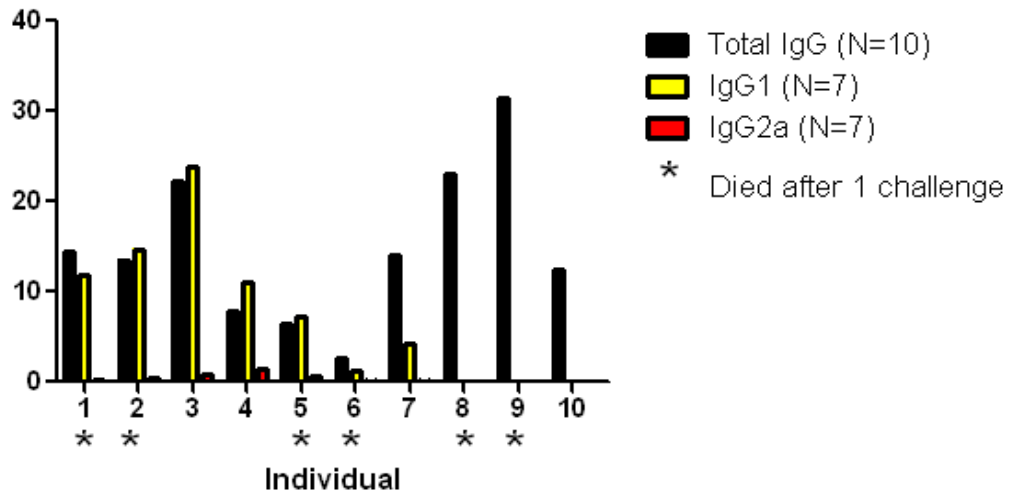


Figure 56: Day 14 *F. novicida* lysate antibody isotype individual mouse levels for Protein + Alum
Plasma samples were isolated from mice after sub-Q vaccination on 0 and 7 days with *F. novicida* lysate (Protein) + alum at 20 µg/mouse. Mice were challenged day 21, 26 and 33 i.p with 232-402 times LD_{50} *F. novicida*. N represents the number of mice. Data is presented as an average. The data highlighted in gray is data where IgG1 and IgG2a data has yet to be tabulated.

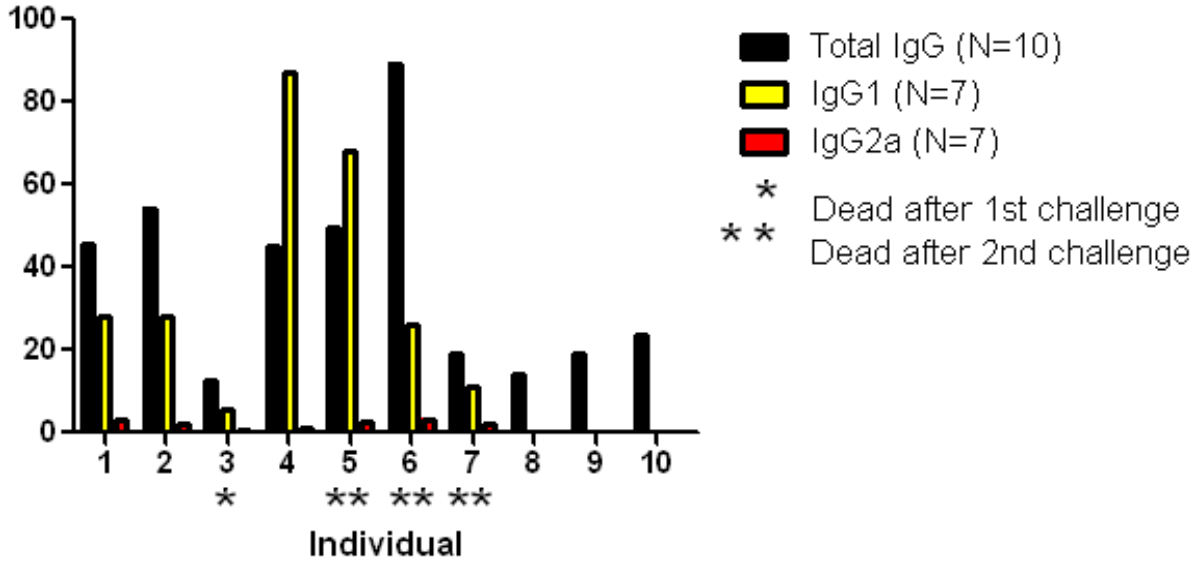


Figure 57: Day 14 *F. novicida* lysate antibody isotype individual mouse levels for Protein/NP
Plasma samples were isolated from mice surviving after sub-Q vaccination on 0 and 7 days with *F. novicida* lysate (Protein) encapsulated in a Ac-DEX nano/micro-particles at 30 mg/mouse. Mice were challenged day 21, 26 and 33 i.p with 232-402 times LD_{50} *F. novicida*. N represents the number of mice. Data is presented as an average. The data highlighted in gray is data where IgG1 and IgG2a data has yet to be tabulated.

7. Live Fire

7.1 Live Fire #1: Misfire

In early October, 2011, 455 mg of bacterial lysate was prepared by the NMRC team as outlined in

6.3.1 except in place of *F. novicida*, an unknown bacterial agent was used. The endotoxin level of this lysate batch, Table 13, was much higher than with the F. *novicida*. Almost simultaneously, NMRC was performing LD50 studies on the same bacteria. Despite i.p. injection of up to $1x10^6$ CFUs, mice were not dying at an appreciable rate, as would be expected for a bacterial pathogen. At this time we worked with DARPA project managers to identify another agent for live fire challenge.

Run	Lysate (mg)	Protein (mg/mL)	LPS (EU/mg)
1	455	6.5	$2.4x10^4$
2	390	6.5	$6.8x10^3$
Pooled	**845**	**6.5**	**$1.6x10^4$**

Table 13: Endotoxin concentration for live fire misfire

7.2 LD50 study of unknowns

In order to perform the live fire challenge, an LD50 of NRMC bacteria stocks were performed. Eleven unknown agents were evaluated for lethality, as outlined in Figure 58. The results of the challenge were given to DARPA program managers by NMRC personnel.

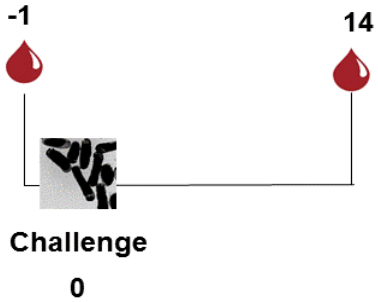


Figure 58: LD50 schedule for Unknowns

7.3 Live Fire #2: Second Attempt

7.3.1 Resiquimod Particle Preparation

Resiquimod particles were fabricated in advance of the live fire challenge. Table 14 reports the mass quantity of particles needed for all experimental conditions listed in Table 4. A scanning electron micrograph of prepared particles is given in Figure 59. The encapsulation efficiency of the resiquimod in the Ac-DEX particles is given in Table 15.

Particle Set	Ac-DEX Needed (gm)
Resiquimod	1.8
Blank	1.8
Lysate	0.8
TOTAL	**3.2**

Table 14: Ac-DEX required for live fire

Batches required for live fire. Approximately five batches of Ac-DEX were reacted to accommodate fabrication of resiquimod, blank and lysate particles for the live-fire challenge.

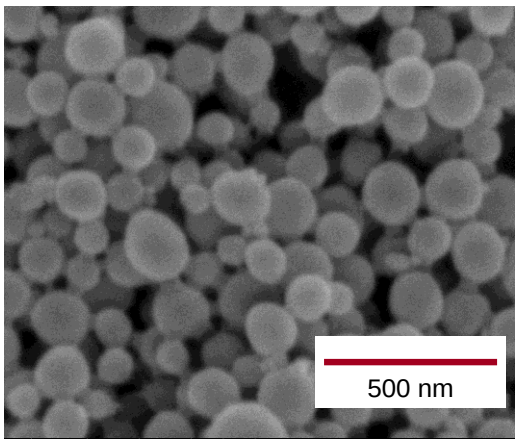


Figure 59: Ac-DEX microparticles encapsulating resiquimod for live fire challenge
Scanning electron micrograph of representative Ac-DEX microparticles encapsulating resiquimod. Scale bar is 500 nm.

Compound	Nominal Loading (wt %)	Encapsulation Efficiency (%)	Quantitative Loading (wt %)	Quantitative Loading (µg Res/mg Ac-DEX)
Resiquimod	3.0	7.33	0.22	2.2

Table 15: Parameters for Live-Fire resiquimod particles

7.3.2 Lysate Preparation

Burkholderia pseudomallei strain 1026b* was selected by DARPA officials for the Live Fire evaluation. Strain 1026b* was isolated in vivo from a mouse that was previously inoculated with B. *pseudomallei* 1026b, forming a more virulent strain of 1026b, not previously published. B. *pseudomallei* lysate was prepared as before and as indicated in Figure 60. The endotoxin concentration of the lysate is given in Table 16. A BCA was performed on the lysate after it was desalted with P10 columns (GE). The results of the BCA are given in Table 17. The total amount of particles required for the live fire is estimated in Table 18.

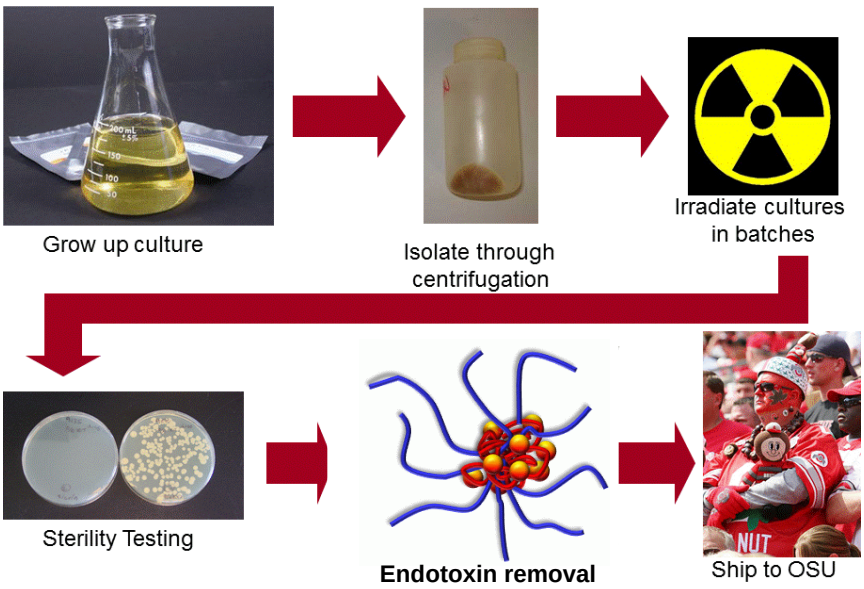


Figure 60: How lysate was prepared for Live Fire Challenge

Run	Lysate (mg)	Protein (mg/mL)	LPS (EU/mg)
Pooled	**105**	**1.0**	**$6.8x10^3$**

Table 16: Endotoxin level of *B. pseudomallei* 1026b* lysate

	Wt loading (mg protein/mg lysate)	Total Protein Amount (mg)	Corrected Endotoxin Level (EU/mg)
Unknown Lysate	0.87	580	$1.2e10^3$

Table 17: Protein Content of Lysate

Particle Set	Total Amount of Particles Needed (mg)
Unknown Lysate	351.7

Table 18: Total Amount of Lysate Particles Needed

7.3.3 Lysate Particle Preparation

Lysate particles were fabricated for the live fire challenge. The amount of particles required is listed in Table 18: Total Amount of Lysate Particles NeededTable 18. The particles were imaged using scanning electron microscopy (Figure 61). The particles were spherical in nature and range in size from approximately 200nm to 2 microns. The loading of the particles was also evaluated. The amount of protein per weight of particle is given in Table 19.

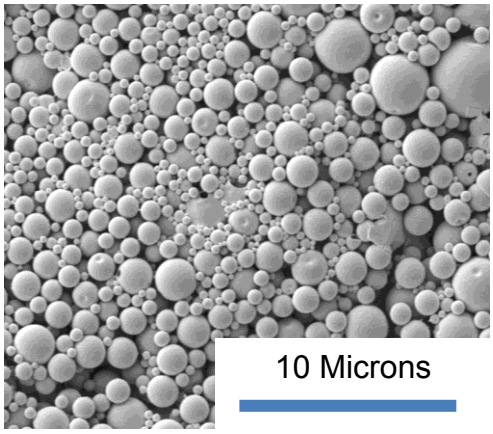


Figure 61: Ac-DEX microparticles encapsulating lysate for live fire challenge.
Scanning electron micrograph of Ac-DEX microparticles encapsulating live fire lysate. The particles were prepared via emulsion chemistry.

Compound	Nominal Loading (wt %)	Encapsulation Efficiency (%)	Quantitative Loading (wt %)	Quantitative Loading (µg Protein/mg Ac-DEX)
Live Fire Lysate	5.0	26.7	0.0134	13.37

Table 19: Characterization of live fire lysate microparticles used for Live Fire challenge.
Nominal loading represents the amount of protein initially added during the formulation process. The Encapsulation Efficiency refers the percentage of the Nominal Loading which remained in the particles after fabrication. The Quantitative Loading the weight percent (wt of drug/wt of Ac-DEX) of protein in the particle. Quantitative Loading is the amount of protein in micrograms normalized to the weight in milligrams of Ac-DEX.

7.3.4 Animal Vaccination

BALB/c mice were vaccinated and challenged (i.p. at an average $1.49x10^6$ CFU of *Burkholderia pseudomallei* strain 1026b*) as per the schedule outlined in Figure 51.

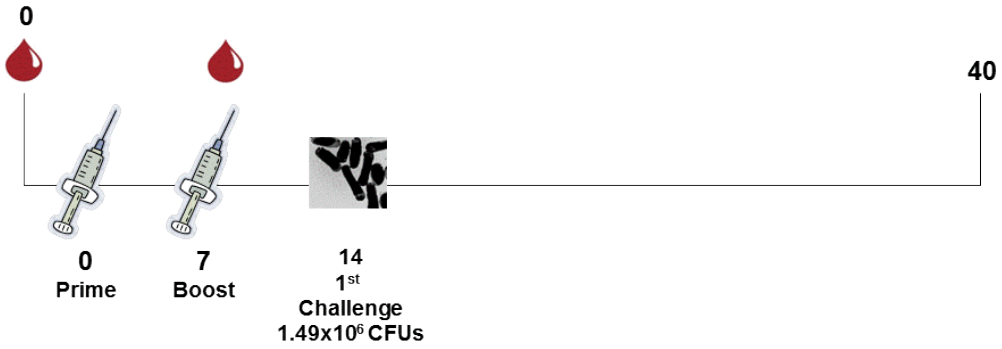


Figure 62: Schedule for Live Fire Test with *B. pseudomallei* strain 1028*
Experimental outline for *B. pseudomallei* strain 1026b* challenge experiment. The numbers indicate the study day in which the indicated procedure was performed. A blood drop represents a collection of blood via lateral tail vein nick. A syringe represents injection of experimental conditions (Table 4) via subcutaneous injection. Challenge is performed by i.p. injection.

7.3.4.1 Mouse Survival with Challenge

The survival of the mice were monitored and reported in Figure 63.

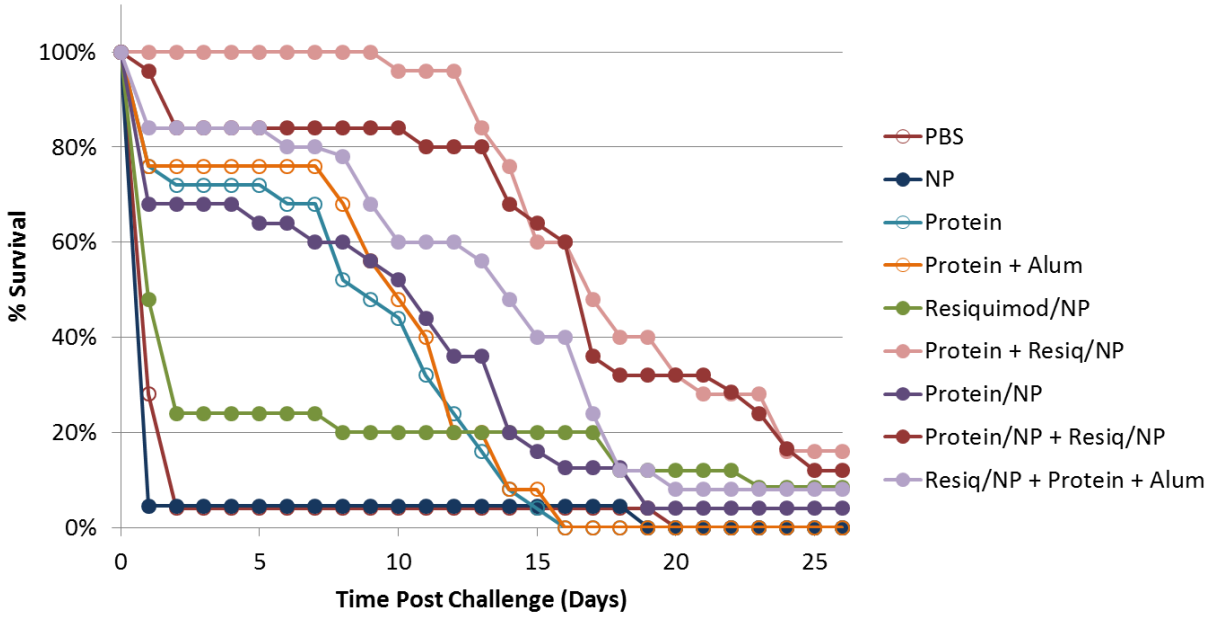


Figure 63: *B. pseudomallei* strain 1026b* survival results
Survival curves of mice given different levels Ac-DEX microparticles with lysate (Protein). Mice were vaccinated on day 0 and 7. The black arrows indicate i.p. challenge (average 235 CFUs).

7.3.4.2 Mouse Bacterial Organ Load

At day 42, the remaining animals were sacrificed, weighed, and their organs harvested, spleens weighed and cultured. The blood samples were plated via direct inoculation of the plate with the samples.

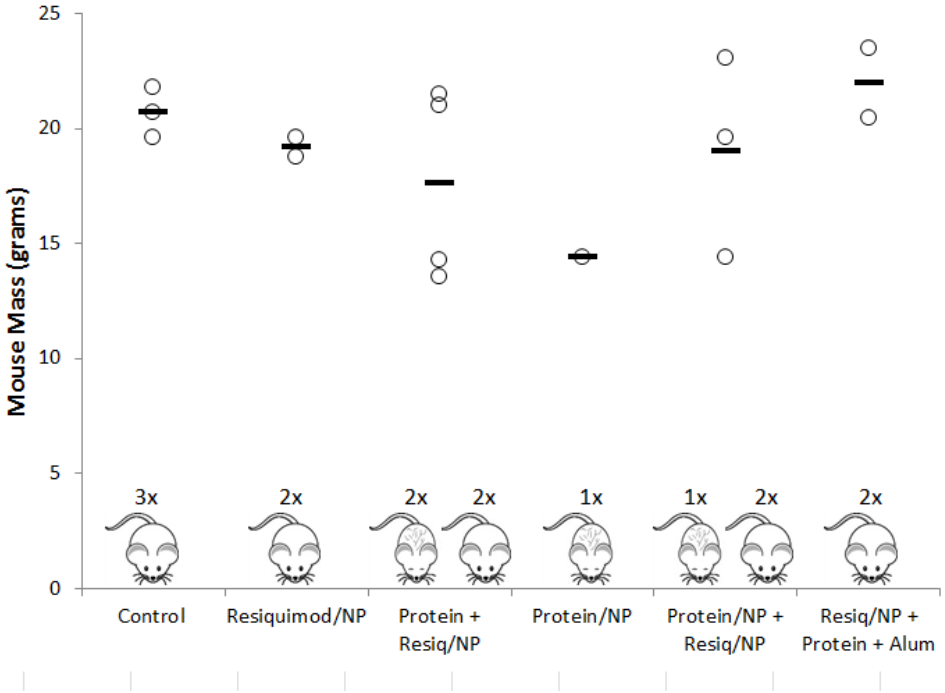


Figure 64: Mouse mass after sacrifice at day 40.
The control group is the PBS control. The numbers above the mice graphic represents the number of mice that were sickly (e.g. squinty eyes, ruffed fur, reduced weight) or mice that were healthy (the left hand mouse graphic where two are presented).

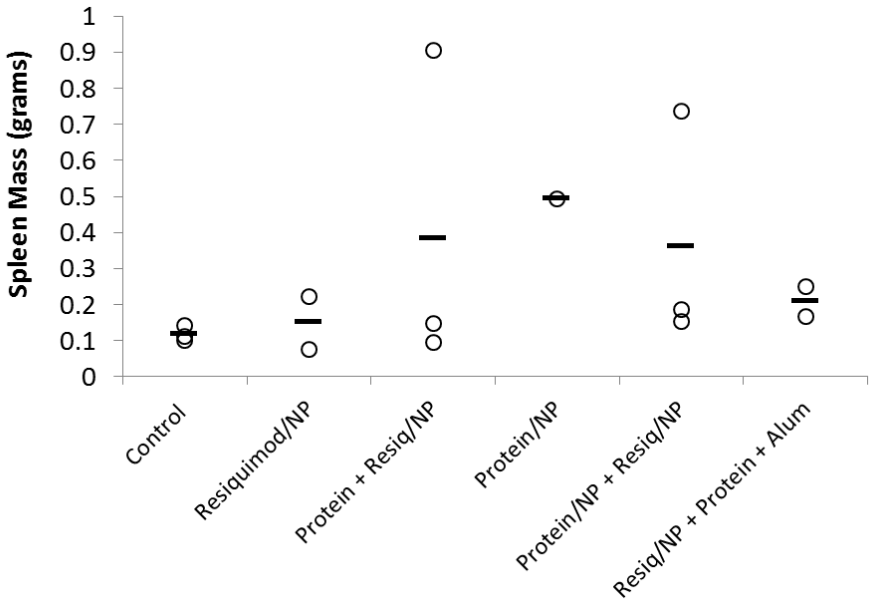


Figure 65: The spleen weights after they were isolated from sacrificed mice.
Only the weights of whole spleens were used. An enlarged spleen is traditionally indicative of an infection in the spleen, resulting in an increased presence of leucocytes.

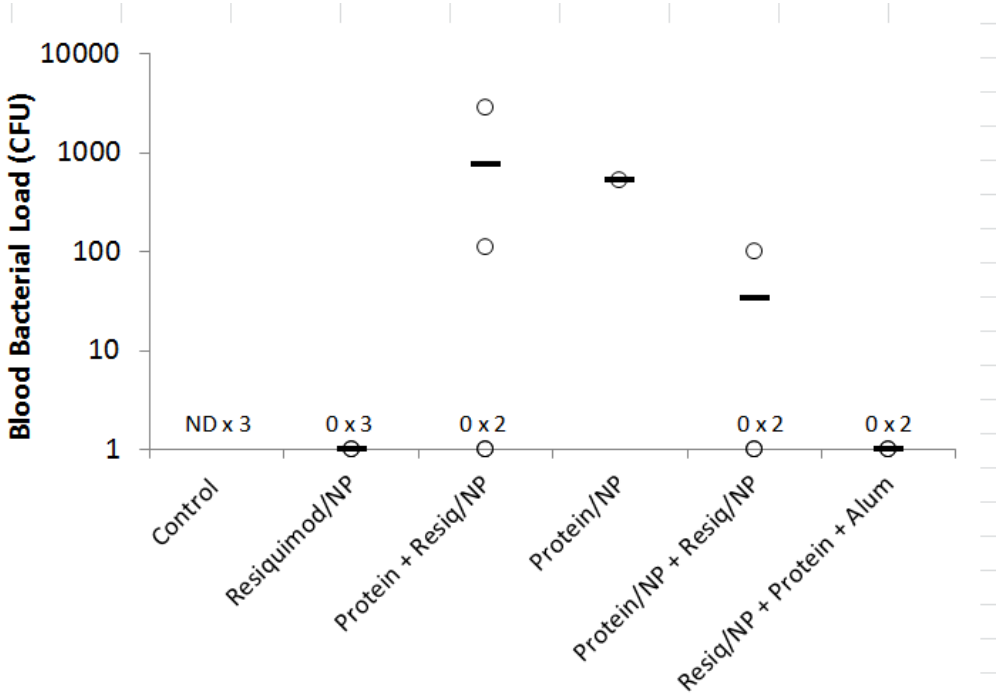


Figure 66: Blood bacterial loads from terminal bleeds isolated on day 40 by cardiac puncture.

ND = Less than detection limits. 0 x n, where n equals the number of mice that had a sterile culture.

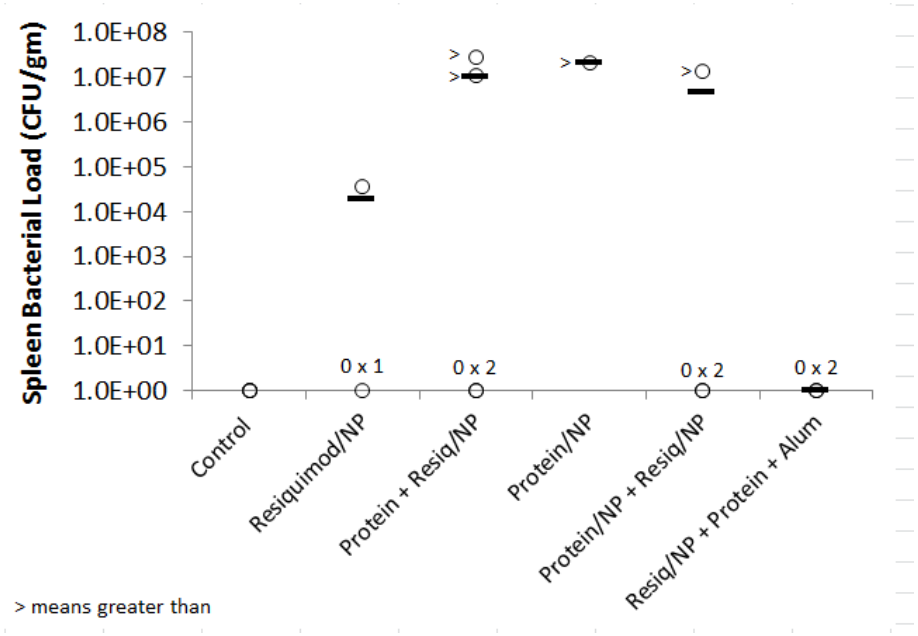


Figure 9: Spleen bacterial loads per mass of spleen on day 40.

0 x n, where n equals the number of mice that had a sterile culture. A > indicates that the count was greater than the indicated value. The average is based on the counts indicated with circle markers.

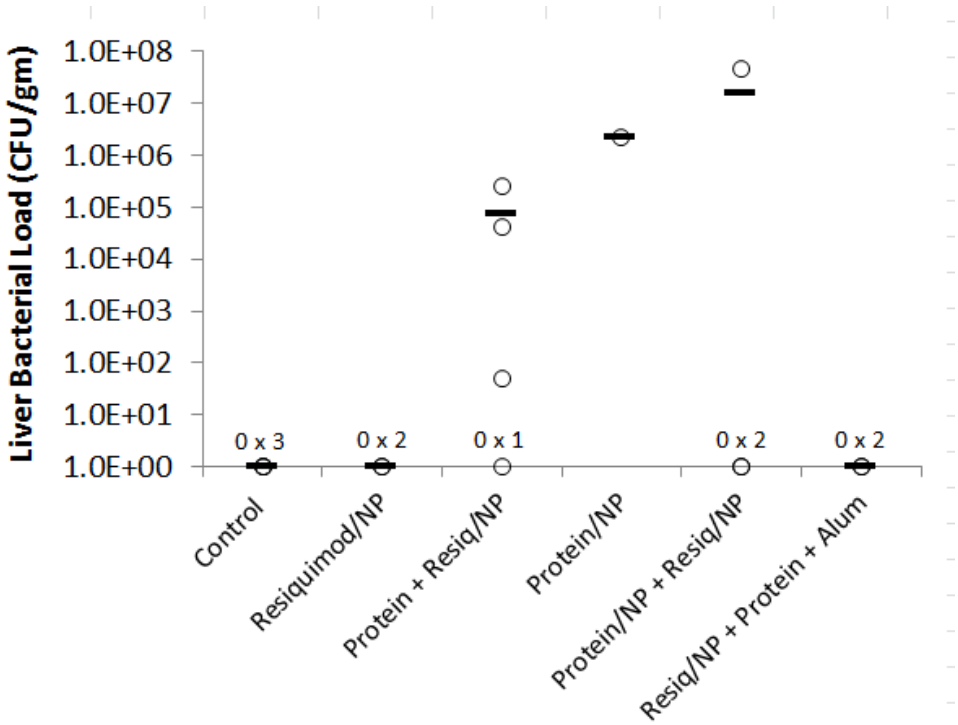


Figure 67: Liver bacterial loads per mass of liver on day 40.
0 x n, where n equals the number of mice that had a sterile culture.

7.3.5 Live Fire Discussion

Table 20 presents a summary of the current state of Melioidosis vaccines. These vaccines cannot be directly correlated to the results of our live fire challenge for multiple reasons:

1. Our bacterial strain (1026b*) was isolated from an animal inoculated with stain 1026b, thereby making it a more virulent strain of *B. pseudomallei* than what has been previously reported. The difference in the bacteria strains, accounts for the misfire experiments mentioned in 7.1 Live Fire #1: Misfire.
2. The timing of our vaccination was aberrant from what is traditionally a vaccine type challenge. Traditionally, a vaccine and challenge experiment is evaluating a memory response for long-term characterization of the immune response. With the experiments outline thus far, our challenges have all been evaluating the effector response. It is hypothesized, that the effector response relies more on the innate immune system for prevention of bacterial infection.
3. Particulate vaccines have yet to be evaluated for Melioidosis prevention. In contrast to solution based vaccines, particulate vaccines would have an extended residence time, target phagocytic cells preferentially, and deliver antigen and adjuvant intracellularly.
4. Resiquimod has not been evaluated before for the vaccination against intracellular bacteria.

In comparison of 1026b strains alone (keeping in mind our strain is more virulent), three of our experimental conditions outperform reported survival. Additionally, our method of vaccination requires less biological purification of antigen, facilitating a more rapid development of the vaccine.

Ref.	Vaccine	Animal model	Vac. route*	Challenge in BALB/c mice model			
				Strain	Route	Dose (CFU)	% survival
78	Lipopolysaccharide	BALB/c mice	i.p.	NCTC4845	i.n.	12.5	0% at d 5
79	LolC (ATP binding cassette system)	BALB/c mice	i.p.	K96243	i.p.	4 x 10^4	80% at d 42
79	LolC (ATP binding cassette system)	BALB/c mice	i.p.	576	i.p.	6.6 x 10^5	30% at d 42
80	Bip proteins	BALB/c mice	i.p.	Ashdown	i.p.	970	0% at d 5
81	Omp3 and Omp7 (Outer membrane proteins)	BALB/c mice	i.p.	D286	i.p.	1 x 10^6	50% at d 21
82	Omp85 (Outer membrane protein)	BALB/c mice	i.p.	D286	i.p.	1 x 10^6	70% at d 15
83	Peptide mimotopes of EPS	BALB/c mice	i.v.	NCTC4845	i.p.	4.7 x 10^4	0% at d 30
84	Hcp2 (integral surface-associated component of T6SS)	Syrian hamster	i.p.	K96243	i.p.	5 x 10^5	83% at d 42
85	Lipopolysaccharide from *B. thailandensis*	BALB/c mice	i.p.	K96243	i.p.	2 x 10^4	50% at d 35
86	Outer membrane vesicle	BALB/c mice	s.c.	1026b	i.n.	5 x 10^3	0% at d 14
86	Outer membrane vesicle	BALB/c mice	i.n.	1026b	i.n.	5 x 10^3	20% at d 14
osu/ nmrc	Lysate + Resqi/NP*	BALB/C mice	s.c.	1026b†	i.p.	1 x 10^6	100% d 10 30% d 20
osu/ nmrc	Lysate/NP + Resqi/NP**	BALB/c mice	s.c.	1026b†	i.p.	1 x 10^6	84% d 11 32% d 20
osu/ nmrc	Lysate + Resqi/NP + Alum**	BALB/c mice	s.c.	1026b†	i.p.	1 x 10^6	72% d 10 12% d 20

Table 20: Comparison of *B. pseudomallei* vaccines.

Table modified from Peacock et al., 2012.[87] * One animal with sterile blood, spleen and liver counts; ** Two animals with sterile blood, spleen and liver counts. † Our strain of 1026b was previously passaged through an animal resulting in higher virulence.

8. Year Two Studies

8.1 Introduction

To elucidate the potential role of resiquimod in the protection observed with previous vaccination models, we were tasked to study the innate response and apoptosis induced by soluble and encapsulated resiquimod.

8.2 Methods

8.2.1 Emulsion Particle Fabrication

Resiquimod (1.5 mg/mL) and Ac-DEX (50 mg/mL) were dissolved in dichloromethane (DCM) and added to an aqueous solution containing 3% w/v polyvinyl alcohol (PVA). The mixture was vortexed for 30 minutes, sonicated for 30 seconds (Misonix Ultrasonic Liquid Processor, 60W, duty cycle 50%) and the emulsion was immediately pipetted into the dispersed phase (0.3% PVA). This solution was then centrifuged (12 min, 17500 rpm, 4 °C), the supernatant was discarded, and the particle sediment was resuspended and washed with basic water (2 x 12 min, 17500 rpm, 4 °C). The microparticles were then suspended in basic water and freeze dried. Blank microparticles were made following the same procedure but without adding adjuvant. PLGA particles were fabricated in the same manner.

8.2.2 Electrospray Particle Fabrication

Ac-DEX microparticles were fabricated using electrospray. Solutions were delivered to a 20 gauge stainless steel capillary (908 μm OD, 603 μm ID) using a programmable syringe pump (KD Scientific, KDS100) at flow rates between 0.1 and 0.3 mL/h. The needle tip was 7 cm above a grounded stainless steel plate (7.6 × 7.6 cm^2) and a high voltage power supply provided the positive high voltage (~4.5 kV) to the stainless steel capillary. Particle collection began once a stable Taylor cone was observed. The collected particles were removed from the plate using a microspatula and stored in a freezer at -20 °C.

Ac-DEX/Tween composite particles (using Tween 80) were all electrosprayed using a flow rate of 0.3 mL/hr and an applied voltage of 4.5 kV. To prepare the electrospray solutions for Ac-DEX/Tween particles, 0.03 mL of Tween was added to 5.97 mL ethanol to make a 0.5% v/v Tween solution. Ac-DEX was then added to produce a solution containing 50 mg/mL Ac-DEX and 5 mg/mL Tween.

Resiquimod loaded Ac-DEX/Tween 80 blended particles were prepared with two drug loadings for drug release and macrophage studies. They were prepared by dissolving resiquimod at levels of 0.5, and 0.05 mg/mL in the Ac-DEX/Tween 80 solutions described above. This yielded solutions where the mass of the drug was 0.9 wt% and 0.09 wt%, respectively, of the total dissolved solute (Ac-DEX and Tween 80). Each solution was electrosprayed for 6 hours and ~50 mg of white powder was recovered. About 50 mg of blank Ac-DEX/Tween 80 particles were also produced.

8.2.3 Scanning Electron Microscopy

The morphology of microparticles was observed by scanning electron microscopy (SEM), using either a Hitachi s-4300 Cold Field Emission SEM or an FEI NOVA NanoSEM 400. Electrosprayed particles were collected on aluminum foil and either imaged directly or suspended in basic water and a small amount was placed on an SEM stub and allowed to air dry. Particle size distributions were measured from SEM images using ImageJ.[88] For a given particle diameter (D_p), the primary droplet size (D_d) was determined using:

$$D_d = D_p \left(\chi \frac{\rho_p}{C_p} \right)^{1/2} \quad \text{Eq. 1}$$

where χ is the particle shape factor (see Equation S.2 in Supplementary Material), ρ_p is the polymer density, and C_p is the polymer concentration in the original solution.

8.2.4 Dynamic Light Scattering

Dynamic light scattering (DLS) measurements (Brookhaven Instruments Corporation, BI 200SM) were made using a laser wavelength of 633 nm, a pinhole setting of 200 μm and a detector angle of 90°. For DLS analysis the particles were collected directly in basic water, and samples were diluted with additional basic water to reduce the average count rate to ~110 kcps.

8.2.5 Preparation of Empty or Resiquimod Loaded Liposomes.

Liposomes were formulated by addition of chloroform and methanol (9:1 v/v) to a mixture of hydrogenated (soy)L-a-phosphatidylcholine, 1,2-distearoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)], cholesterol (Ovine Wool), D-alpha-tocopherol (Acros Organics, Morris Plains, NJ), either with or without resiquimod (Enzo Life Sciences, Farmingdale, NY). The lipid solution was rotary evaporated with a Buchi R-200 rotary evaporator (New Castle, DE) with a Buchi B-490 water bath set at 60 ^{0}C. Lipids were reconstituted in dd-H2O for 30 minutes in a 60 ^{0}C water bath. Liposomes were freeze-thawed 3 times, followed by extrusion through an Avanti Mini-Extruder/Heating Block with an 80 nm polycarbonate membrane and filter supports (Alabaster, Al), 11 times before passage through a disposable PD-10 column (GE Healthcare, Pataskala, OH). Sucrose was added to the liposomes (150 wt %) followed by lyophilization.

8.2.6 Encapsulation Efficiency and Drug Loading

The encapsulation efficiency (EE) of resiquimod is defined as the mass ratio of resiquimod to polymer in the particles divided by the mass ratio of resiquimod to polymer in the initial electrospray solution, given as a percent. Resiquimod fluorescence (Ex: 260nm/Em: 360nm) was quantified using a Spectra Max Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). Three separate samples of particles containing resiquimod and three blank particle samples were dissolved in DMSO at 1 mg/mL. Each sample was pipetted in triplicate into a 96 well plate and read using the microplate reader. The concentration of resiquimod was determined by using a calibration curve.

8.2.7 Culture of RAW Macrophages

RAW macrophages (ATCC Manassas, VA) were cultured as outlined by manufacturer's directions.

8.2.8 Nitric Oxide Release

NO release was quantified using Griess reagent (VWR International, Radnor, PA). Macrophages were seeded at 45,000 cells/well and incubated at 37°, 5% CO_2 for 18 hours. The media was then replaced with media containing resiquimod particles or free resiquimod and incubated at 37°, 5% CO_2 for 24 hours. After incubation, the supernatant was removed using centrifugation and the Griess assay was performed according to the manufacturer's specifications.

8.2.9 Cell Viability

Macrophage viability was determined using 3-[4,5-dimethlthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). MTT was dissolved in media at a concentration of 5 mg/mL and added to the macrophages at a concentration of 20 μL/well. Viable macrophages formed formazen crystals inside the cell that were dissolved using isopropanol and the resultant color quantified by its absorbance at 560 nm.

8.2.10 Apoptosis

Macrophage apoptosis was calculated using the TUNEL Assay (R&D Systems, Minneapolis, MN) and Annexin V (Life Technologies, Pittsburgh, PA). Macrophages were seeded at 45,000 cells/well and incubated at 37°, 5% CO_2 for 18 hours. The media was then replaced with media containing resiquimod

particles or free resiquimod and incubated at 37°, 5% CO_2 for 24 hours. After incubation, the cells were washed and the assay performed according to the manufacturer's specifications.

8.2.11 *Burkholderia thailandensis* culture, inoculation and resiquimod treatment

Burkholderia thailandensis (ATCC) were grown on Luria-Bertani (LB) agar plates or in LB broth with aeration at 37°C. For infection bacterial strains were grown to mid-logarithmic phase by diluting overnight cultures 1:20 and growing them to an optical density of 1.7 to 2 at 600 nm. Macrophage inoculation and treatment is depicted in Figure 68.

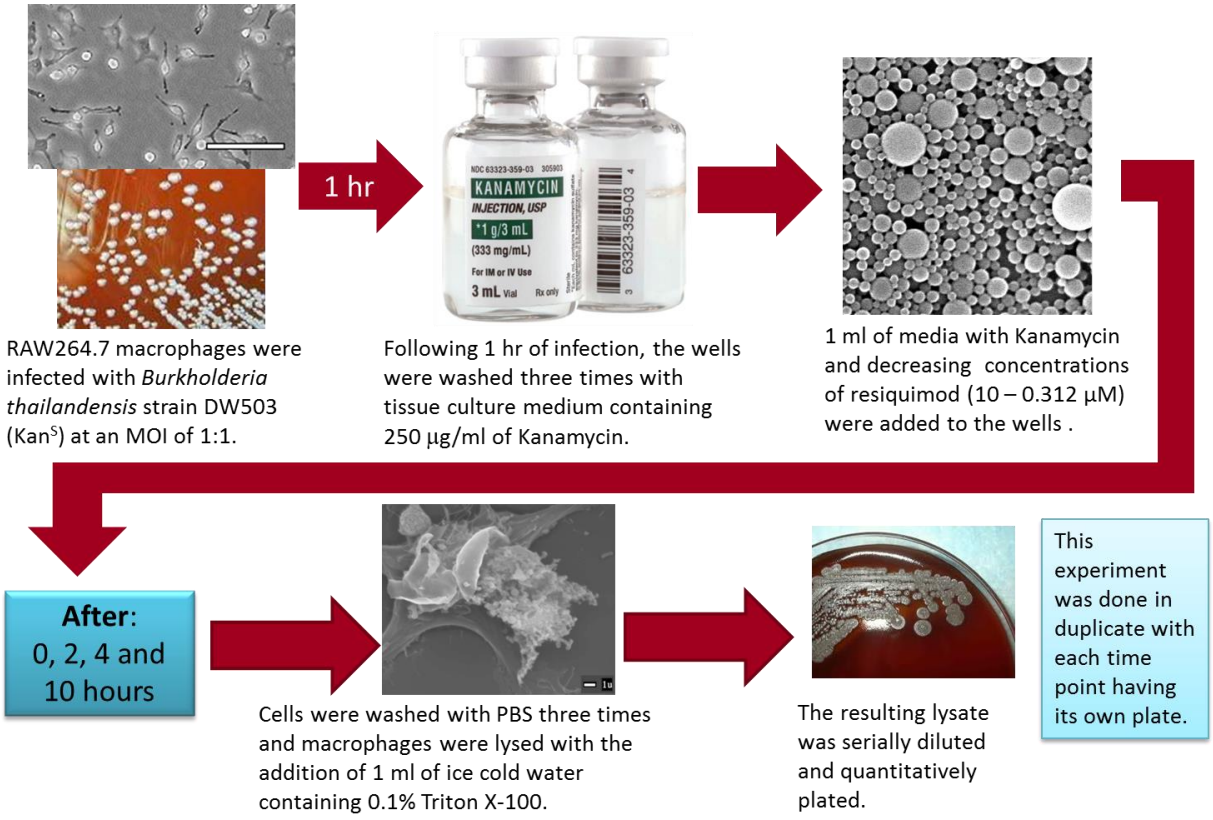


Figure 68: Schematic of *B. thailandensis* inoculation and treatment with resiquimod

8.3 Results and Discussion

8.3.1 Particle morphology

To evaluate the size and shape of the polymeric particles, scanning electron microscopy was used.

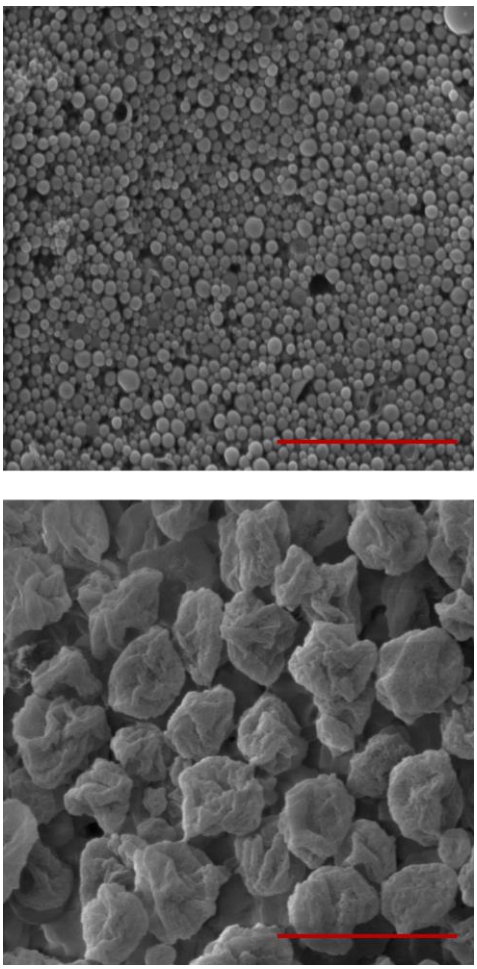


Figure 69: Scanning electron micrographs of Ac-DEX particles encapsulating resiquimod

Ac-DEX particles can be made via emulsion (top) or electrospray (bottom) techniques. These particles share similar mechanisms of action but differ in properties such as shape, size, and encapsulation efficiency. Scale bar represents 3 µm.

8.3.2 Encapsulation Efficiency and Particle Sizing

The loadings and encapsulation efficiencies of all formulations is presented in Table 21.

Particles	Loading	Encap Eff	Size (nm)
Emulsion Ac-DEX	3%	8.71%	203.8 ± 100
Blank Emulsion Ac-DEX	NA	NA	216.7 ± 116.9
Emulsion PLGA	3%	1.57%	221.6 ± 22.5
Blank Emulsion PLGA	NA	NA	249.7 ± 39.6
Electrospray	0.22%	73.63%	839.4 ± 291.5
Blank Electrospray	NA	NA	839.8 ± 206.3
Liposomes	5.60%	5.57%	1193 ± 1012
Blank Liposomes	NA	NA	215 ± 93

Table 21: Encapsulation efficiencies and size of all formulations used to encapsulate resiquimod

8.3.4 Innate Immune Response

Figure 70 and Figure 71 show the observed nitric oxide (NO) production and cell viability in macrophages treated with increasing doses of free and encapsulated resiquimod. Figure 70 shows that for encapsulated drug at the low loading, the NO production initially increases with resiquimod dose, reaching a maximum at doses of 0.016 and 0.031 μg/mL. All doses of encapsulated drug (except for the lowest dose) showed significantly higher NO production than the blank particle control. For the high drug loaded particles, the NO production was significantly greater than that for free resiquimod at every dose level, with the exception of 0.031 μg/mL (where there was a large variance in replicates) and 0.004 μg/mL (the lowest dose).

For the low loaded particles, the NO production was significantly higher than that for free drug at lower doses of 0.008 and 0.016 μg/mL, while the opposite is observed at higher doses. Figure 70 (b) shows that relative to the media only control, there was no significant effect of free resiquimod, compared to medium only, on the cell viability over the concentration range investigated. Moreover, the presence of blank particles does not show a significant effect on cell viability compared to media only. However, when compared to free drug at the same resiquimod dose, cell viability was significantly lower when cells are exposed to particles containing resiquimod at low drug loading for each of the three highest doses.

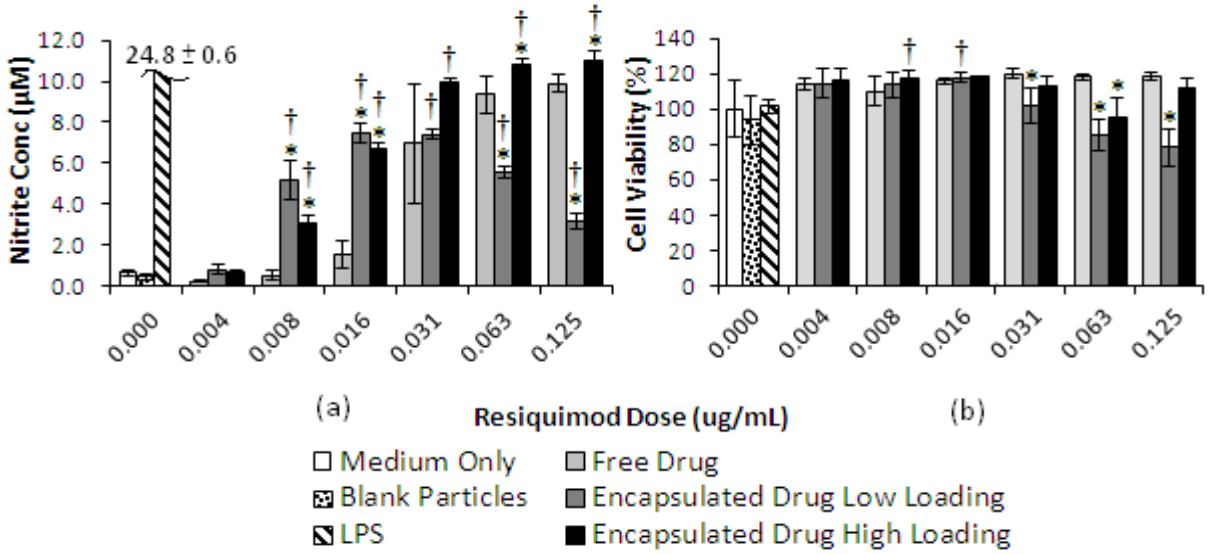


Figure 70: Nitric oxide production and cell viability for electrosprayed resiquimod particles
(a) Nitrite production and (b) cell viability of RAW macrophages treated with free and encapsulated resiquimod. Both graphs show results from one experiment run in triplicate (n=3). Error bars represent one standard deviation. An * indicates significant difference with respect to free drug at the respective dose ($p < 0.05$). A † indicates significant difference with respect to blank particles ($p < 0.05$).

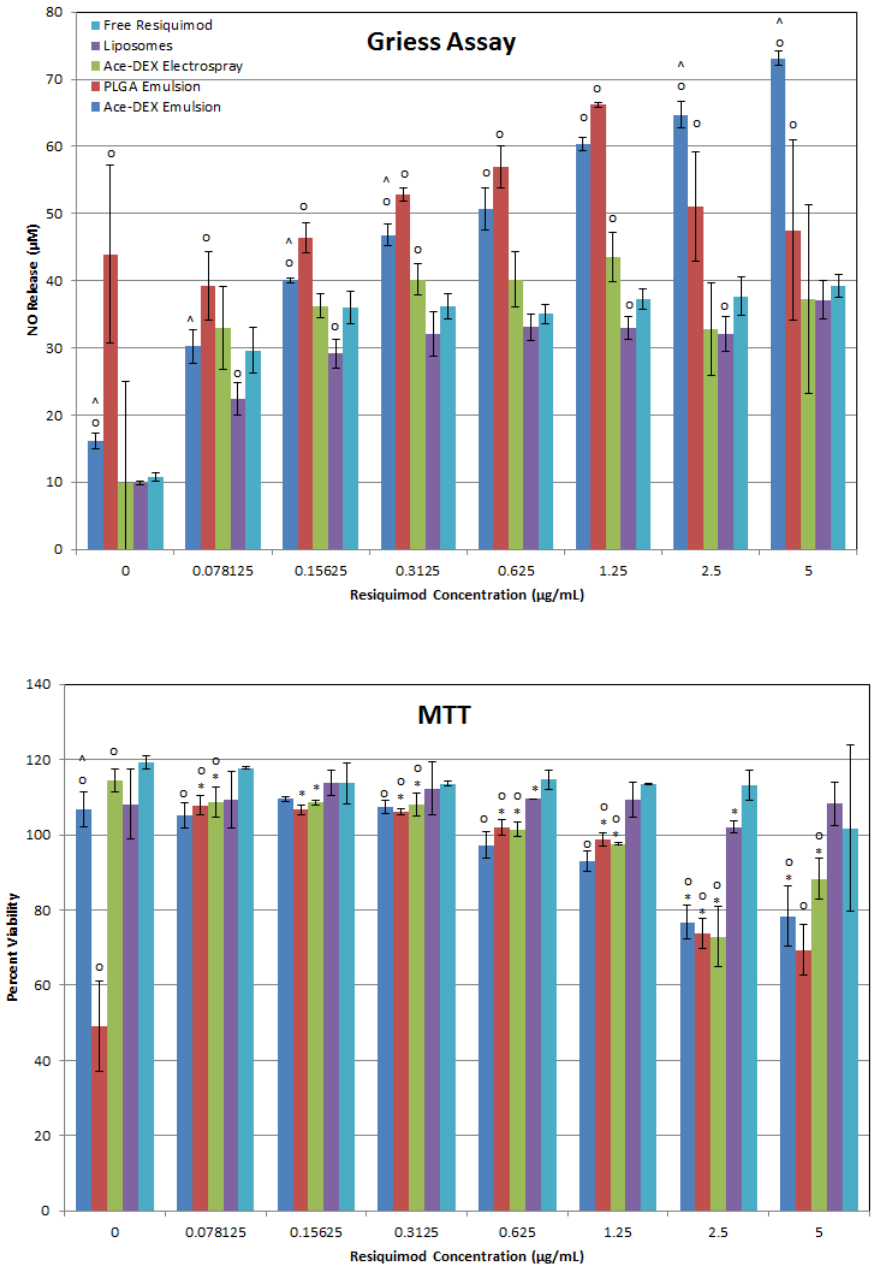


Figure 71: Nitric oxide and viability of cells cultured with various formulations encapsulating resiquimod

(Top**)** NO release and (bottom) cell viability results compare the safety and efficacy of resiquimod-loaded particles. * $p<0.05$ vs. respective blanks, º $p<0.05$ vs. free drug, ^ $p<0.05$ vs. PLGA.

The production of three inflammatory cytokines (TNF-α, IL-6, and RANTES) is shown in Figure 72. Although we also measured the production of IL-1β, MIP-1α and IL-12p70, these results are shown in Figure 73 because they are either too low to be considered practically significant (IL-1 β and IL-12p70), or they lack statistical significance compared to free drug or blank particle controls (MIP-1α).

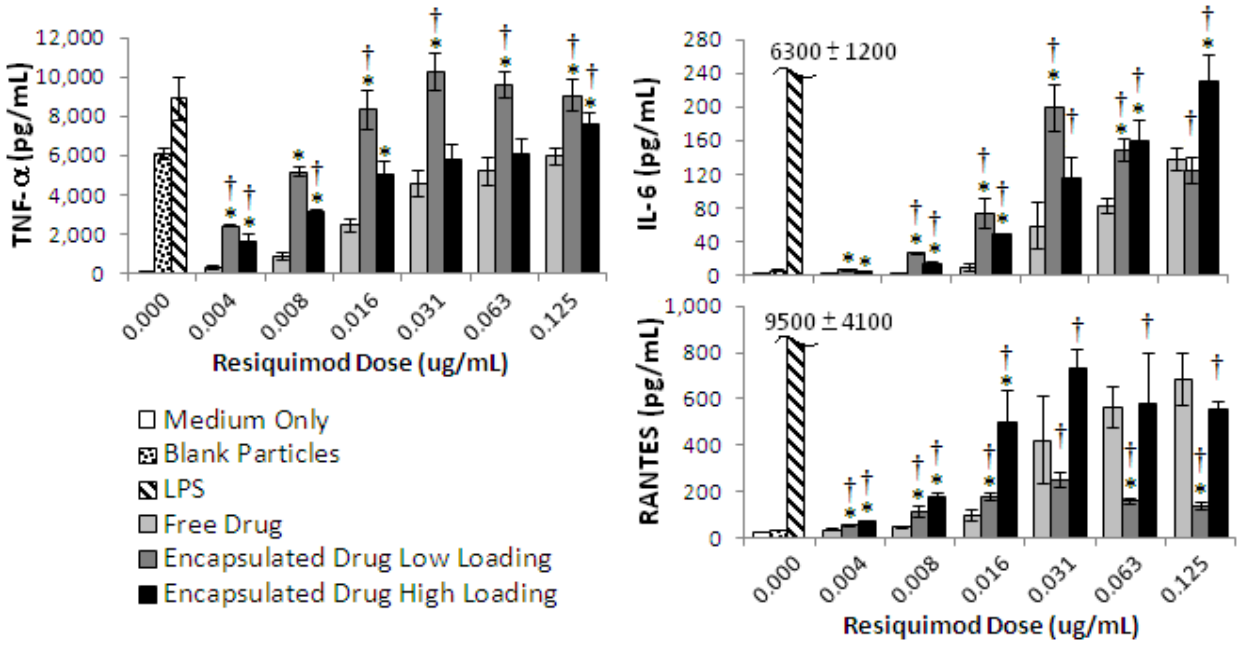


Figure 72: Inflammatory cytokine production in RAW macrophages treated with free and encapsulated resiquimod.

All graphs show cytokine results from triplicate wells. Error bars represent one standard deviation. An * indicates significant difference with respect to free drug at the same dose ($p < 0.05$). A † indicates a significant difference relative to the blank particle control ($p < 0.05$).

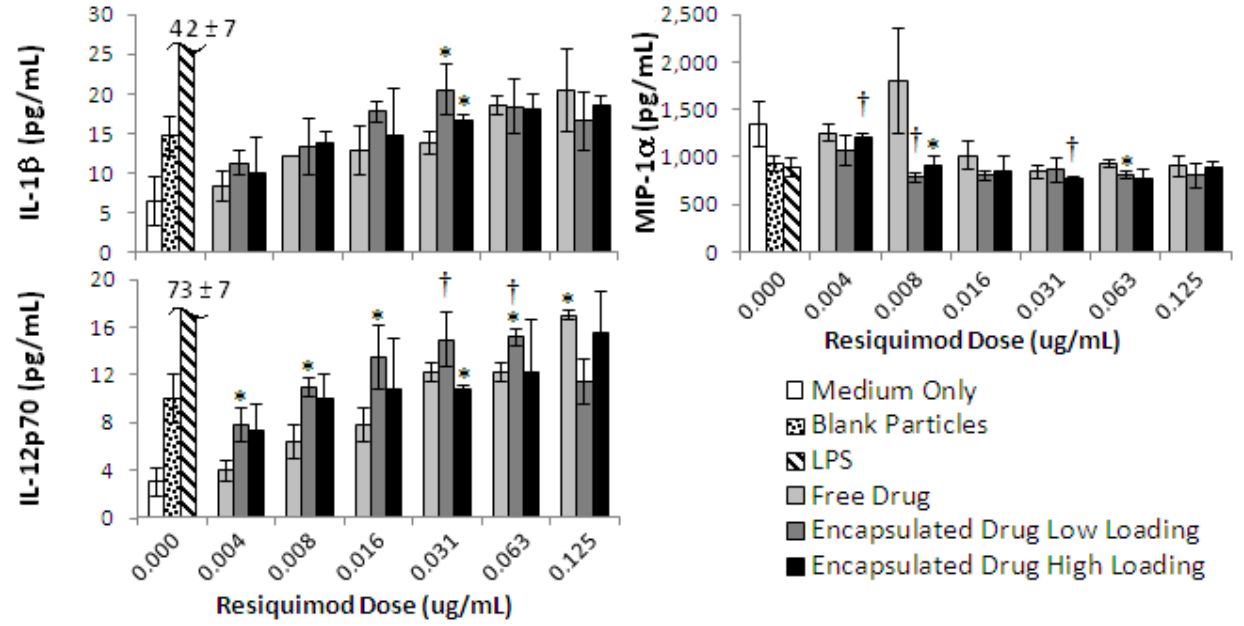


Figure 73: Concentration of three inflammatory cytokines that were shown to be insignificant.

These concentrations are either too low to be considered practically significant (IL-1 β and IL-12p70), or lack statistical significance compared to free drug or blank particles (MIP-1α). MIP-1α concentrations are below the background concentration in cells grown in pure medium. An * indicates significant difference with respect to free drug at the same dose ($p < 0.05$). A † indicates a significant difference relative to the blank particle control ($p < 0.05$).

8.3.5 Apoptosis of Macrophages with Resiquimod Treatment

Macrophages cultured with resiqumod formulations were evaluated for apoptosis that results in DNA damage (Figure 77; TUNEL) or phosphatidylserine expression during late stage apoptosis (Figure 77; Annexin V).

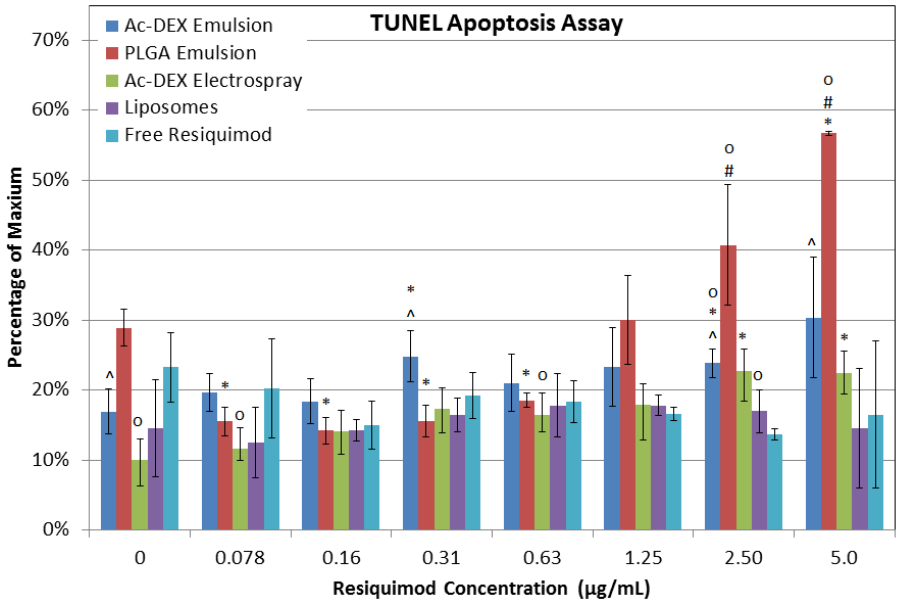


Figure 74: Apoptosis of Macrophages Cultured with Resiquimod Formulations evaluated through a TUNEL assay

Apoptosis induction as compared to untreated macrophages. Zero concentration was calculated using blank MPs at the highest particle concentration. * $p<0.05$ vs. respective blanks, º $p<0.05$ vs. free drug, ^ $p<0.05$ vs. PLGA, # $p<0.05$ vs. untreated cells

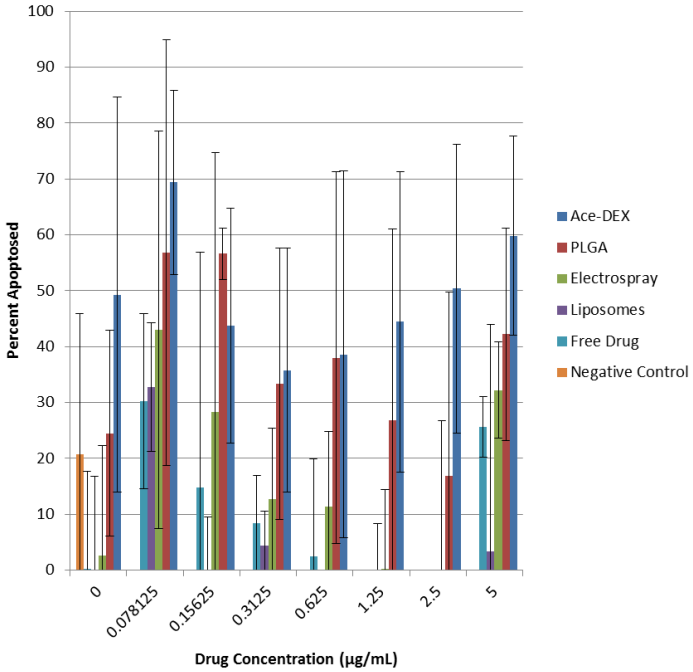


Figure 75: Late State Apoptosis of Macrophages Cultured with Resiquimod Formulations evaluated through Annexin V fluorescent staining

8.3.6 Treatment of *Burkholderia thailandensis* infection with resiquimod

Macrophages were inoculated with *B. thailandensis* (Figure 68) and treated with various concentrations of resiquimod. Resiquimod treatment significantly reduced CFUs even at the lowest concentrations.

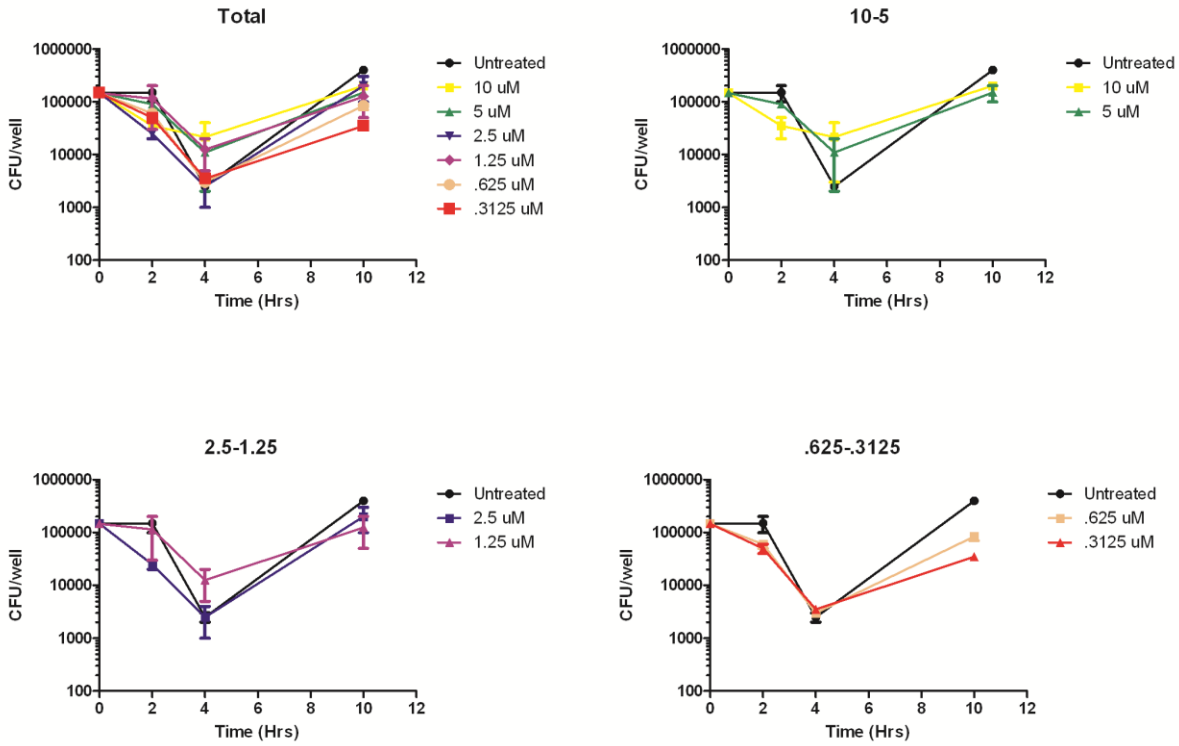


Figure 76: Effect of soluble resiquimod treatment on B. thailandensis infected macrophages

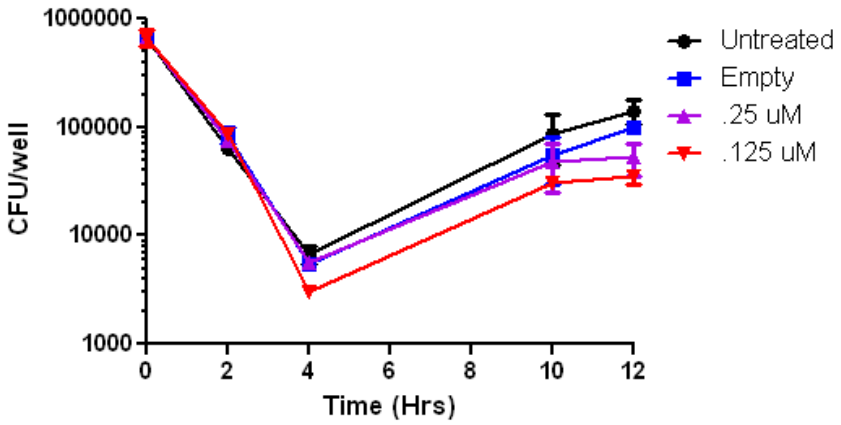


Figure 77: Effect of Ac-DEX electrosprayed particles encapsulating resiquimod on B. thailandensis infected macrophages

8.4 Conclusions

Our results indicate that apoptosis is not a significant factor for the vaccine response observed during year 1 of the project, where emulsion resiquimod particles were used. Of all the formulations evaluated, electrosprayed encapsulation seems to be the most tolerable and potent. Both soluble and encapsulated resiquimod significantly inhibit *Burkholderia* growth in macrophages.

9. Draft *Francisella Novicida* Paper

Rapid protection against *Francisella novicida* via vaccination with acetalated dextran microparticles encapsulating cell lysate and resiquimod

Kevin L. Schully[1], Sadhana Sharma[2], Kevin J. Peine[2], John Pesce[1], Margret A. Elberson[1], Mariko. E. Fonseca[1], Angela M. Prouty[1], Matthew G. Bell[1], Eric M. Bachelder[2], Andrea Keane-Myers[1], Kristy M. Ainslie[2*]

[1]Vaccine and Medical Countermeasures Department, Biological Defense Research Directorate, Naval Medical Research Center, Silver Spring, MD 20910, USA
[2]College of Pharmacy Division of Pharmaceutics, The Ohio State University, Columbus, OH 43210, USA

Corresponding author:
Kristy M. Ainslie, PhD
500 W 12th Ave.
242 LM Parks Hall
Ohio State University
Columbus OH 43210
Phone: 614-688-3797
Fax: 614-292-7766
Email: Ainslie.1@osu.edu

Abstract

Introduction

For the lethal infection tularemia, caused by *Francisella tularensis (F. tularensis)*, the time from exposure to death is less than the time required to create an effective humoral or cell-mediated immune response. Therefore, the development of vaccine formulations that can generate a rapid immunity are needed to combat potential bioterrorism attacks from this Center for Disease Control (CDC) Biodefense Category A Priority Pathogen. *F. tularensis* is an aerobic gram negative coccobacilli, that is considered a class A bioterrorism agent due to its ease of discemination, high mortality rate, low infectious dose, and potential for social disruption.[70] There are four known species in the *Francisella* family: *tularensis, holarctica, medisiatica,* and *novicida*. *Tularenis* (Type A) is found primarily in North America, and is associated with rabbits and ticks while *holarctica* is typically associated with ticks and mosquitoes, and it produces a reduced clinical phenotype with decreased infection and mortality rates.[71] *Novicidia* is a closely related to *F. tularensis* and has been classified as a BSL2 sub-species of the lethal bacteria. At least six people worldwide have been infected with *F. novicidia*[89] and it is a commonly used variant to initially evaluate *F. tularensis* vaccine preparations.[90]

Several formulations have been developed in an attempt to create and effective *F. tularensis* vaccine. A live vaccine strain (LVS) of *F. tularensis* has been developed for protection of humans.[91] Even though LVS has been used for many years for vaccine applications, it has not been licensed by the FDA due to many reasons including the incomplete knowledge of the mechanism of attenuation, and residual virulence that is present after administration. Safer versions have been tested including crude culture extracts and subunit proteins, but both methods have failed to produce protection.[92,93] Both methods are probably inefficient due to the complex immune response that is necessary to protect against highly virulent strains[94]. Furthermore, in the development of a rapid response vaccine, it is likely that activation of the innate immune response is vital, particularly for combating *F. tularensis.* Several studies have shown that even though the host is able to recognize *F. tularensis,* infection results in poor

immune stimulation, and in fact actively induces immune suppression by reducing the host's ability to respond to pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS).[76] Clearly new vaccine formulations are needed to combat ***Francisella*** species.

The safest vaccine formulations are those which are subunit or protein based. Sub-unit vaccines, however, suffer from lack of immunogenicity. To combat reduced immunogenicity, a toll-like receptor (TLR) agonist can be co-delivered. Freely delivered TLR agonists, however, may result in a myriad of complications ranging from autoimmunity to systemic toxic.[95,96,97] Our lab has successfully encapsulated several adjuvants that are either only available in FDA approved topical formulations (e.g. imiquimod, resiquimod)[27,98] or for pre-clinical application (e.g. poly I:C, CpG).[99] For encapsulation, we use the novel polymer acetalated dextran (Ac-DEX). Ac-DEX is a dextran based materials that has tunable degradation rates leading to the pH neutral degradation products of dextran, ethanol and acetone, a common metabolic byproduct.[18,100] For the release of adjuvants, we have shown that acid-sensitive Ac-DEX enhances the delivery of the drug resulting in a dose sparing effect compared to free drug and significantly greater cellular activation compared to the ubiquitously used polymer, poly(lactic-co-glycolic acid) (PLGA).[27,98,99] For the delivery of protein based antigens, we have also showed significantly enhanced CD8 presentation of peptide with Ac-DEX compared to other biomaterials, including PLGA.[18] This has translated in to effective formulation of a rapid anthrax vaccine, that conferred protection from triplicate challenged, seven days post vaccination and one week apart, after two vaccinations, also one week apart.[98] To evaluate Ac-DEX microparticles as a universal platform for vaccination, we developed a *F. novicida* vaccine comprised of bacterial lysate and the TLR 7/8 FDA approved agonist resiquimod. We evaluated the antibody titer, and antibody sub-class.

Materials and Methods

Chemicals (Sigma, St. Louis, MO) were used as received, unless otherwise noted.

Ac-DEX Synthesis

10 or 71 kDa dextran was used to react acetalated dextran (Ac-DEX, 59% cyclic acetal coverage).[27,101-103] After freeze drying, dextran was dissolved in DMSO with the catalyst pyridinium p-toluenesulfonate and reacted with 2-methoxypropene for five minutes or four hours, quenching with triethylamine (TEA). The product was purified with basic water (Nanopure wate with TEA; pH 9) under vacuum, and freeze dried. For purification, the product was then dissolved in ethanol, centrifuged (10 min, 10,000 x *g*, Beckman RA-21, Los Angeles, CA, USA), precipitated in basic water and freeze dried to yield purified Ac-DEX.

Ac-DEX NMR Analysis

Ac-DEX's spectrum was recorded with a 300 MHz ^{1}H-NMR (Bruker 300 Ultrashield). The cyclic acetal:acyclic acetal ratio, which determines the degradation rate, was concluded from the NMR spectra.[18] The Ac-DEX used for the encapsulation of *F. novicida* lysate was 10K MW dextran reacted for four hours (cyclic coverage of 79.5%) and for encapsulation of resiquimod, 71K MW dextran reacted for five minutes was used (cyclic coverage of 59.2%). These different MWs were used as then maximize the encapsulation efficiency of the respective compound.

Resiquimod Emulsion Particle Fabrication

Resiquimod was purchased from Alexis Biochemicals, Enzo Life Sciences (Farmingdale, NY). Ac-DEX and resiquimod were dissolved in dichloromethane (DCM; 3 mg resiquimod/100 mg Ac-DEX), combined with 3% PVA in phosphate buffer solution (PBS), vortexed for 2 minutes and sonicated for 30 seconds (Misonix Ultrasonic Liquid Processor, 60W, duty cycle 50%). The emulsion was added with the aqueous phase (0.3% PVA) that was spinning on a stir plate. This was then centrifuged (12 min, 17500 rpm, 4 °C) after three hours, the supernatant discarded, and the particle sediment resuspended in basic water. The particles were washed three times in total with basic water to remove excess drug and lysate. The microparticles were then suspended in basic water and freeze dried without additional compounds added (e.g.

cryoprotectants). Blank microparticles were made following the same procedure but without adding adjuvant.

Culture and Generation of *F. novicida* lysate

Figure S.1 presents a schematic of lysate preparation and Table 1 reports the endotoxin results. We grew *F. novicida* in Mueller-Hinton broth supplemented with1 % isovitalex (BD) to an OD_{600} of approximately two. The bacteria was isolated via centrifugation and resuspend the pellet in HEPES. After resuspension, we bacteria was lysed with a microfluidizer set at 20,000 p.s.i. Cell wall protein and other contaminants were removed through centrifugation. Protein was isolated by ammonium sulfate precipitation and desalted. After isolation, the protein content was quantified and endotoxin was removed via Triton X-114 micellar removal. Endotoxin removal was performed in batches and for each batch the process was performed at least once. The final endotoxin concentration is reported in Table 2.

Lysate Emulsion Particle Fabrication

Initially the protein content of the lysate was characterized using a bicinchoninic acid assay (BCA assay; Thermo Fisher Scientific, Rockford, IL) with albumin as a standard. Since the BCA cross-reacts with dextran, protein concentration was also determined with a fluorescamine (4-phenyl-spiro [furan-2(3H), 1'-phthalan] -3,3' -dione) assay[104], as it would be later used to calculate the lysate encapsulation in microparticles. Figure S.2 presents the standard curve of the lysate based on the protein concentration of the lysate determined by the BCA.

For encapsulation, lysate was dissolved in PBS and added to a DCM and Ac-DEX solution (1.5 mg lysate/100 mg polymer). The initial emulsion was homogenized for 30 seconds at 20k rpm using Polytron PT 10-35 Homogenizer (Westbury, NY), added to a 3% PVA solution, and homogenized again for 30 seconds. This was immediately added to 0.3% w/w PVA/PBS solution and stirred for 3 hours and treated as above with the resiquimod particles.

Encapsulation Efficiency:

Resiquimod's encapsulation efficiency (EE) was calculated via fluorescence (Ex: 260nm/Em: 360nm) after being dissolved in DMSO, using a Spectra Max Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA). Lysate EE was determined using a fluorescamine assay [104] after Ac-DEX was degraded in PBS and 50-vol% formic acid nanopure water mixture (pH 3.0) at 37°C for 24 hours. For analysis, the pH was adjusted to 7.4 with a NaOH solution.

Scanning Electron Microscopy

SEM micrographs were collected using a FEI NOVA NanoSEM 400. To prepare the samples, a 10 mg/mL solution of particles in basic water was added to a silicon wafer (Ted Pella; Redding, CA) and the samples were left to air dry. After sputter coating with a layer of gold alloy for 120 seconds the samples were analyzed with SEM.

Vaccination

Weighted out lyophilized particles were placed in microcentrifuge tubes and immediately before injection the particles were suspended in 200 microliters of PBS to reach a final lysate and adjuvant concentration of 15 or 8 micrograms per mouse. For groups with alum, Imject (Thermo Scientific) was used for experimental conditions with alum (20 mg/ml (4 mg per a mouse)). The appropriate suspension was then drawn into a 1 ml syringe and 200 µl was injected subcutaneously into the scruff of the mouse with a 25 gauge needle. Mice were vaccinated at day 0 and 7 in the same location.

Anti-lysate IgG Detection

Mice (n=10, Experimental Groups Table 2) were vaccinated on day 0 and 7 as outlined above. The lateral tail vein of each mouse was nicked at fixed times points (-3, 14, and 30 days) and 50 microliters of blood was collected from each mouse. On day 42, mice under deep anesthesia, blood was removed via intracardiac puncture. Whole blood was allowed to clot at room temperature and then serum was separated from the clot by centrifugation and stored at -80 °C.

To determine anti-lysate antibody concentration, Microtiter plates (Immulon-IV HBX,Thermo Labsystems, Franklin, MA) were coated with lysate (1µg/mL) and incubated overnight at 4°C in 0.2 M carbonate buffer (pH7.4). Following blocking and washing, serum samples isolated from vaccinated mice were incubated with the lysate coated plates. After washing, a secondary anti-mouse IgG horseradish peroxidase-conjugated anti-mouse IgG anti-bodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was then incubated at room temperature with the plate. The plates were then washed and developed with ABTS one-component substrate (KPL),stopped with the addition of 1% SDS and the absorbance at 405 nm was read with a

standard ELISA plate reader. Readings in the linear range were compared to the quantitative standard curve to determine by anti-Protective Antigen (PA) IgG concentrations (µg/ml).[105] (IgG antibody subclass (IgG1 or IgG2a) was determined from the day 42 terminal blood samples by ELISA as described above except that standard curves were generated with mouse IgG1 and

IgG2a subclass controls (Invitrogen).

Antigen-specific restimulation of splenocytes

Antigen-specific restimulation of splenocytes (antigen recall assay) was performed in a manner to what has been previously outlined.[105] Breifly, the spleen was pushed through a 100 µm nylon cell strainer (Fisher) into a six-well tissue culture plate containing 5 ml of tissue culture medium [RPMI 1640+GlutaMax with penicillin-streptomycin (5 ml/500 ml), L-Glutamine (5 ml/500 ml), 10% Hyclone FBS heat inactivated (56° for 30+ min, 50 ml/500 ml), and 0.1% 2-Mercaptoethanol (0.5 ml/500 ml)], to create a single cell suspension. After isolation of cells through centrifugation, red blood cell lysis buffer was added and the suspension was centrifuged again. Viable cells were counted with a hemocytometer and cultred in a 96-well plate. Cells were stimulated with Concanavalin A (5 µg/ml) or lysate (12 µg/ml) in RPMI for 72 hours. Stimulated cytokine production was detected in the supernatants using a BioPlex Pro

(BioRad) custom cytokine kit that included IL-2, IL-6, IL-17 and IFN-γ; and were measured on a BioRad Luminex-200 according to the manufacturer's instructions

Results and Discussions

Particle Characterization

Ac-DEX particles were fabricated to encapsulate *F. novicida* lysate and resiquimod. Scanning electron micrographs of the particles are given in Figure 1. Particles are less than 1 micron in size and can easily be phagocytosed by dendritic cells once injected into the subcutaneous environment.[106,107] Particles larger than 100nm are not easily taken up by non-phagocytic cells and will be passively targeted to phagocytes.[106,107] The particle size observed in Figure 1 indicates that although there is a large polydispersity, as is common to emulsion particles, almost all the particles fall between 100nm and 2 microns accommodating uptake by primarily phagocytes. To calculate the encapsulation efficiency (EE) of the lysate protein particles, a standard curve with lysate protein using the fluorescamine assay (Figure S.2) was developed. The encapsulation efficiency of the particles is given in Table 2. The encapsulation of bacterial lysate was on the order of what was previously shown with ***Helicobacter pylori*** encapsulation in PLGA (62-75% EE with 4% loading)[108] and *Streptococcus equi* lysate into PLGA (80% EE).[109] The encapsulation of resiquimod in polymeric particles, to the best of the authors' knowledge, has only been performed by the authors of this manuscript[105,110]. Other work in the Ainslie lab has indicated that greater EEs of resiquimod can be achieved with electrospray generation of particles[111], however emulsion was used in this publication. The low EE of resiquimod occurs because of the partitioning of the drug into the aqueous continuous phase. A higher EE was observed with imiquimod[27], a parent compound of resiquimod, because it is significantly more hydrophobic and does not partition well into the aqueous phase. Resiquimod however, is a more potent cytokine activator than imiquimod, and that is why it was chosen for this study.[112]

Cytokine production with antigen recall

Figure 2 reports the cytokine production of splenocytes isolated from vaccinated mice (0 and 7 days) and exposed to antigen (antigen recall). In general, macrophage inflammatory protein (MIP)-1α and interleukin-17 (IL-17) have little significance between the experimental groups. On the other hand, some trends regarding statistical comparison to experimental conditions Lysate/MP + Resq/MP and Resiq/MP + Lysate + Alum can be gleaned from the data. For both of these groups, IL-1β, IL-2, IL-6, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) are all significantly higher compared to encapsulated lysate. Furthermore, the IL-12(p70) protein level is significantly lower for encapsulated lysate compared to Lysate/MP + Resiq/MP, although the overall level of cytokine production is quite low and probably negligible for all experimental conditions. Insignificant levels of cytokines were also detected with TNF-α.

Two (IL-2 and IFN-γ) of the cytokines significantly upregulated in the Lysate/MP + Resq/MP and Resiq/MP + Lysate + Alum groups play predominant roles in antigen specific T cell responses, whereas IL-1β and IL-6 are thought to enable proliferation of T cells and other lymphocytes.[113,114] Indicating that perhaps a strong antigen specific T cell response occurs in these vaccinated mice, over lysate + alum. With a rapid vaccination schedule, other research has shown that a strong CD8 response occurs. Peng et al. report an antigen specific CD8+ response can be generated by a plasmid-based cancer vaccine with a shortened vaccination schedule. Their response was shown to reduce cancer volume[115]. Preliminarily, one might consider that the presence of encapsulated TLR-7/8 agonist resiquimod would induce the marked difference between groups Lysate/MP + Resq/MP and Resiq/MP + Lysate + alum, and Lysate + alum, since alum responses are understood to be Th2, primarily.[113,114] Since the Lysate + Resiq/MP group has levels near the other groups (and certainly not significantly different from all groups, except Lysate/MP + Resq/MP and Resiq/MP + Lysate + Alum), it would appear the presence of resiquimod is not the sole contributor to this differential cytokine profile and the Lysate/MP + Resq/MP and Resiq/MP + Lysate + alum groups appear to have

synergistic elements that account for the increase in cytokine production. This synergy could lie in the combination of encapsulated antigen and TLR agonist, as particulate formulations of antigen have been shown to provide CD8+ stimulation[18,29,116] over unencapsulated formulations (e.g. with alum) that result in primarily CD4+ response.

Humoral response to lysate antigen and rapid vaccination schedule

Rapid vaccination and immunity is needed for a number of lethal infections (including Tularemia), since the time course from pathogen exposure to death is shorter than the duration of time required to create an effective humoral immune response. To evaluate rapid immunity with our vaccine formulation, A/J mice were vaccinated sub-cutaneously at 0 and 7 days. Additionally, in an effort to evaluate the application of our polymer formulation in developing a rapid universal vaccine platform, bacterial lysate was used as an antigen, since it can be grown easily and quickly after a bacterium is isolated from an exposure event. This in combination with the rapid vaccination schedule could be applied to develop a protective vaccine quickly in the wake of release of an unknown biodefense agent.

Serum samples taken before and after vaccination were analyzed for lysate specific antibody concentration (Figure 3A). The antibody concentration for the conventional lysate + alum formulation was not significantly different from the encapsulated lysate (Lysate/MP) concentration; however, the fraction of total IgG that was IgG1 and IgG2 in the two groups were significantly different (Figure 3B). IgG1 antibodies have a high affinity for their protein target and are a T-cell helper 2 (Th2) immune mediated response. In contrast, IgG2 antibodies are generated during a Th2 response, are less susceptible to proteolytic degradation than IgG1 and have a moderate affinity for their protein.[113,114] The combination of IgG1 and IgG2 antibodies have shown to be protective against a lethal intranasal *F. novicida* challenge in BALB/c mice. The authors concluded that a Th1 bias was needed, in addition to B cells, to confer protection against a lethal challenge.[117] Other studies have indicated that protective immunity against *F. tularensis* is medicated through IFN-γ as well as TNF-α.[117] It appears that with our current

formulation, a high level of antibody titer and these crucial cytokines are not present in any one of the nine formulations evaluated. Additional studies will need to be performed to better evaluate a viable antigen for protection against *Francisella Sps.* In a mapping of immunoreactive antigens from patients suffering from Tuleremia, whole cell lysate and membrane proteins were shown to have the most immunorelevant antigens after LPS.[118] Other studies have indicated that anti-LPS is the primary antigen for LVS inoculated mice.[118] Antigen isotype mapping could be performed fully elucidate the formed antibodies. Additionally, perhaps either better endotoxin purification of the whole cell lysate or isolation of membrane proteins could result in a better outcome for application of this platform as a vaccine against *Francisella* infection.

Conclusions

The development of rapid antigen specific immunity is needed to combat bioterrorism attacks, such as could be carried out with Category A agent *Francisella Tularensis*. We have formulated a lysate based particulate vaccine that illustrates antigen specific responses and could provide protection against a lethal *Francisella* challenge shortly after vaccination. The conventional subunit formulation of protein (lysate) with alum illustrated a strong antibody response as well as potent cytokine expression with antigen recall in comparison with the encapsulated formulations. Overall additional studies need to be performed to determine the survivability of mice after a lethal challenge and also determine if protective cytokines and antibody responses are better generated by using lysate with decreased endotoxin content.

Run	Lysate (mg)	Endotoxin (mg/mL)	Endotoxin (EU/mg)
1	40	7.3	30.3
2	40.5	8.1	124
3	29.5	8.2	13.4
Pooled	**110**	**7.8**	**58.9**

Table 1: Endotoxin level of *F. novicida* lysate

Phosphate Buffer Solution (sham)	PBS	0	0	0
Blank microparticles	MP	0	0	0
Unencapsulated Lysate	Lysate	15	0	0
Unencapsulated Lysate with Alum (traditional formulation)	Lysate + Alum	15	0	4
Encapsulated Resiquimod	Resiq/MP	0	8	0
Free Lysate and Encapsulated Resiquimod	Lysate + Resiq/MP	15	8	0
Encapsulated Lysate	Lysate/MP	15	0	0
Encapsulated Lysate and in separate microparticles, encapsulated Resiquimod	Lysate/MP + Resiq/MP	15	8	0
Encapsulated Resiquimod, Free Lysate and alum	Resiq/MP + Lysate + Alum	15	8	4

Table 2: Experimental groups for all in vivo studies. The experimental groups, abbreviations, protein amount, resiquimod amount and alum concentrations are given.

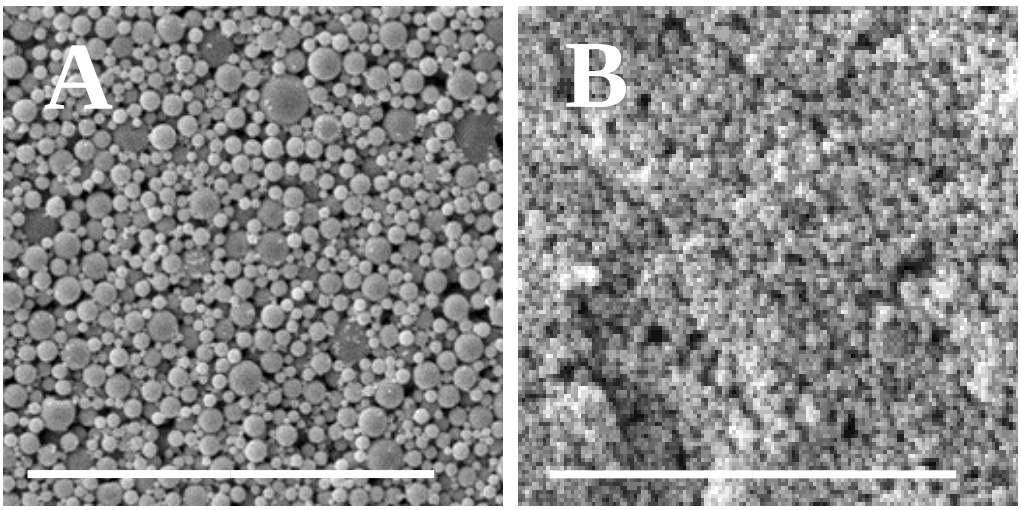


Figure 1: Scanning electron micrograph of representative Ac-DEX microparticles encapsulating (A) *F. novicida* lysate and (B) resiquimod. Scale bar is 10 microns.

Particle Set	Wt loading (mg/100mg Ac-DEX)	%Efficiency
***F. novicida* Lysate**	1.70 ± 0.14	47.3 ± 3.8
Resiquimod	0.22 ± 0.00037	7.4 ± 0.012

Table 3: Encapsulation characteristics of Ac-DEX microparticles encapsulating *F. novicida* lysate or resiquimod. Initial resiquimod loading is 3 mg drug/100 mg Ac-DEX and *F. novicida* lysate protein 5 mg lysate/100 mg Ac-DEX.

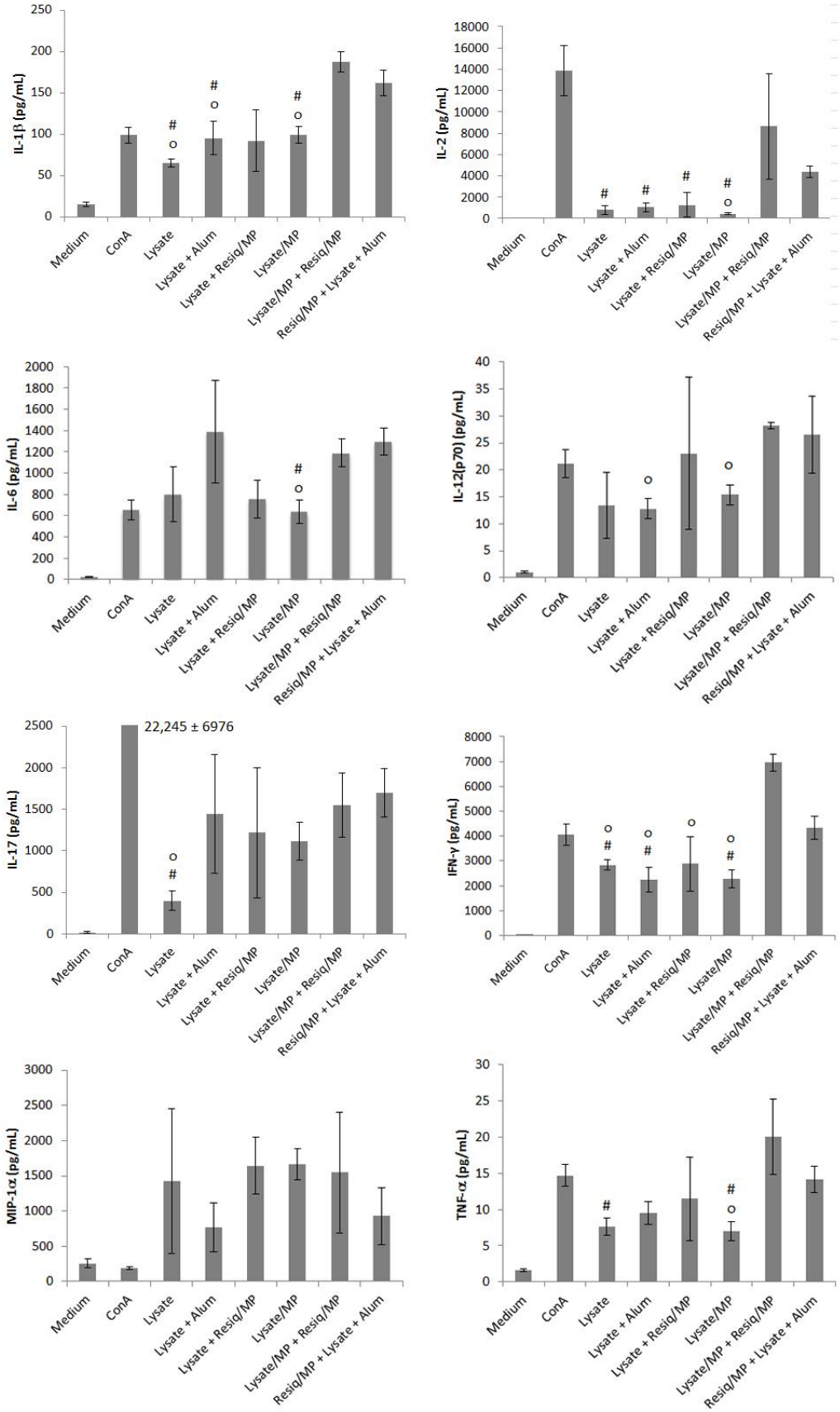

IL-1β (pg/mL)
IL-2 (pg/mL)
IL-6 (pg/mL)
IL-12(p70) (pg/mL)
IL-17 (pg/mL)
22,245 ± 6976
IFN-γ (pg/mL)
MIP-1α (pg/mL)
TNF-α (pg/mL)
Medium
ConA
Lysate
Lysate + Alum
Lysate + Resiq/MP
Lysate/MP
Lysate/MP + Resiq/MP
Resiq/MP + Lysate + Alum

Figure 2: Cytokine production for *F. novicida* lysate recall assay with splenocytes isolated from mice vaccinated at day 0 and 7 with indicated experimental conditions. Encapsulated condition is indicated with /MP. Resiq indicates 8 ug/mouse of resiquimod. Lysate is *F. novicida* lysate at 20 µg/mouse. ConA represents Concanavalin A, which is a lectin used as a positive control. Data is presented as average ± standard deviation. All detected cytokines for all experimental conditions were significantly different (P-value < 0.5) from the concentration found in media alone except MIP-1α compared to ConA. An # indicates significance (P-value < 0.05) with respect to Lysate/MP + Resiq/MP, an o indicates significance with respect to Resiq/MP + Lysate + Alum and an * indicates significance with respect to lysate + alum.

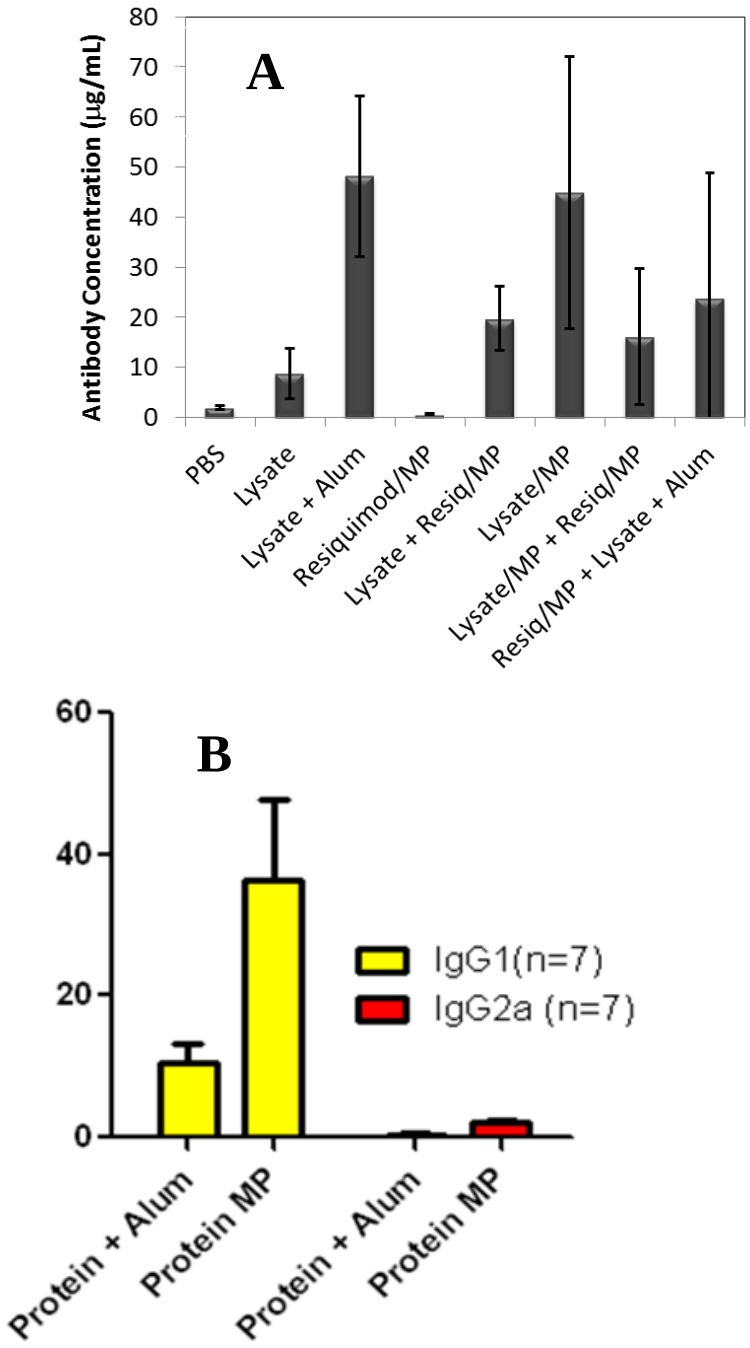


Figure 3: *F. novicida* Lysate specific antibody concentration for blood samples taken from mice (A/J, n=3) on day 14 after being vaccinated on day 0 and 7 with indicated experimental condition. (A) Presents total IgG concentration and (B) presents IgG subclass. Data is presented as average ± standard deviation.

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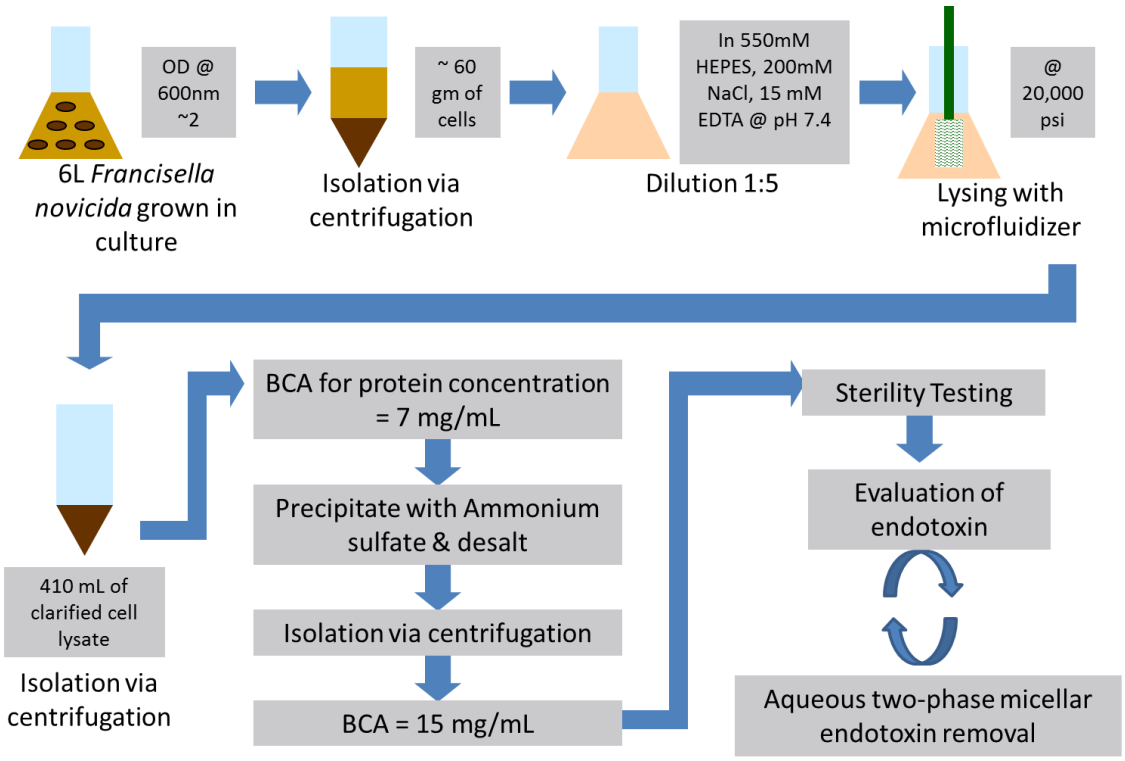

Figure S.1: Preparation of *F. novicida* lysate wherein endotoxin is removed via Triton X-114 solution.

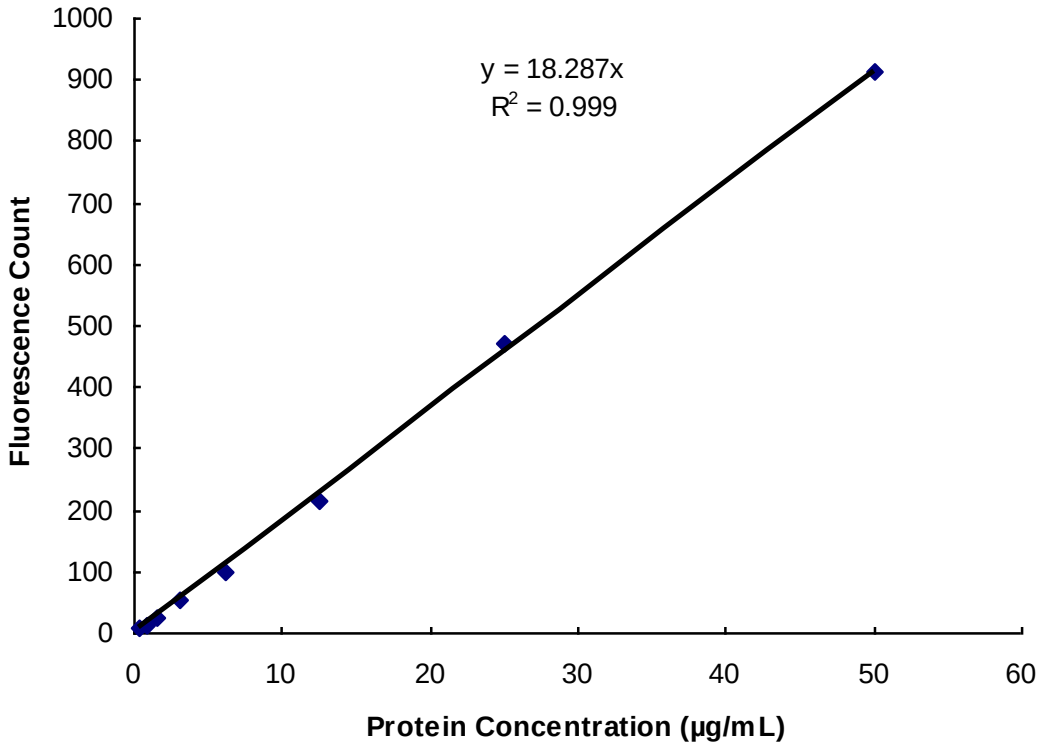

Figure S.2: Standard curve of *F. novicida* lysate measured with fluorescamine assay. Fluorescamine assay detects lysine groups on proteins. The standard curve was used to calculate encapsulation efficiency.

10. Presented Works

10.1. Peer Reviewed Publications

1. Schully KL, Sharma S, Peine KJ, Pesce J, Elberson MA, Fonseca ME, Prouty AM, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. Rapid vaccination using an acetalated dextran microparticulate subunit vaccine confers protection against triplicate challenge by bacillus anthracis. Pharm Res. 2013 May;30(5):1349-61.
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3. Schully KL, Sharma S, Peine KJ, Pesce J, Elberson MA, Fonseca ME, Prouty AM, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. Rapid protection against Francisella novicida via vaccination with acetalated dextran microparticles encapsulating cell lysate and resiquimod. In preparation.
4. Schully KL, Sharma S, Peine KJ, Fonseca ME, Bell MG, Borteh H, Gallovic M, Bachelder EM, Keane-Myers A, Ainslie KM. Microencapsulated vaccine for rapid and memory protection against highly virulent strain of *Burkholderia pseudomallei*. In preparation.
5. Brackman DJ, Gallovic M, Schully KL, Peine KJ, Borteh H, Bachelder EM, Keane-Myers A, Ainslie KM. Encapsulated resiquimod for *Burkholderia thailandensis* infection. In preparation.

10.2 Oral Presentations

1. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. OMICS Vaccines & Vaccination, Chicago, IL, July 2013.
2. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. AIChE, Pittsburgh, PA, Nov 2012.

10.3 Poster Presentations

1. Kevin L. Schully, Sadhana Sharma, Kevin J. Peine, John Pesce, Margret A. Elberson, Mariko. E. Fonseca, Angela M. Prouty, Matthew G. Bell, Eric M. Bachelder, Andrea Keane-Myers, Kristy M. Ainslie. Acetalated dextran microparticles for the enhancement of rapid response vaccine for bacillus anthracis. AIChE, Washington, DC, Nov 2013.
2. Deanna J. Brackman, Hassan M. Borteh, Matthew D. Gallovic, Kevin J. Peine, Eric M. Bachelder, Kristy M. Ainslie. Encapsulated Resiquimod as a Treatment for Visceral Leishmaniasis. The Ohio State University College of Pharmacy Research Day, Columbus, OH. 2013
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